Elucidation of spatiotemporal variations in functional genes involved in the nitrogen cycle along the west coast of India

A thesis submitted to Goa University for the award of degree of

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In

MICROBIOLOGY

By

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CERTIFICATE

Certified that the research work embodied in this thesis entitled "**Elucidation of spatiotemporal variations in functional genes involved in the nitrogen cycle along the west coast of India**" submitted by Ms. Jasmine Gomes for the award of Doctor of Philosophy degree in Microbiology at Goa University, Goa, is the original work carried out by the candidate himself under my supervision and guidance.

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DECLARATION

As required under the University ordinance, I hereby state that the present thesis for Ph.D. degree entitled "**Elucidation of spatiotemporal variations in functional genes involved in the nitrogen cycle along the west coast of India**" is my original contribution and that the thesis and any part of it has not been previously submitted for the award of any degree/diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area.

The literature related to the problem investigated has been cited. Due acknowledgement have been made whenever facilities and suggestions have been availed of.

JASMINE GOMES

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I take this privilege to express my heartfelt thanks to everyone involved to complete my Ph.D successfully. First and foremost, I want to thank my Lord Jesus Christ for His favour throughout my PhD. I did not join NIO with the intention of Ph.D., but when my Boss encouraged me to register for the same, I prayed about it and decided to give a try, and the journey begins from there. I express thanks to Jesus because it was He alone, who cleared me in the 2013 August PhD entrance exam at the very first attempt, when I had absolute no confidence in me, and I knew He wanted me to continue. Later when i was out of tenure and I needed fellowship to continue my PhD, He chose me to be the Senior Research Fellow (SRF) that I could never imagine being selected in life sciences without His favour. So without Him I would have never been able to complete my thesis. Thank you my Jesus. All praise, honour and glory to my Lord Jesus Christ for His grace, mercy, peace and blessing me with the following wonderful people in accomplishing this thesis.

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Dedicated to My beloved

LORD JESUS CHRIST

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Chapter 1

GENERAL INTRODUCTION

1.1 <u>Introduction</u>

When viewed from space, it is evident why our planet would be better named "Ocean" than "Earth." More than 70 % of the planet's surface is covered by interconnected bodies of water that include lagoons, salt marshes, intertidal zones, estuaries, mangroves, coral reefs, the deep sea, sediments, hydrothermal vents among others. The open ocean, all its water columns and all basins -shallow to deep hadal- provide the largest habitable opportunity in particular for microbes, and adoptable biota, in general.

Simply stated, the marine ecosystem is complex, confluent and composite. This continuum of water body encompasses the neritic to offshore, pelagic to abyssal, intertidal to hadal habitats. The Earth's largest ecosystem is complex in its physical (physiography, temperature, pressure, color, light) and chemical (elemental, gaseous and isotopic) characteristics. Seawater, with over 95 known elements dissolved in it, is chemically the most complex fluid. Within this exist myriad, teeming and thriving life forms adapted to inhabit a variety of ecotypes. The inhabitant biota of immense diversity spanning from lysogenic virions to gigantic blue whales has evolved, adopted and existed from early millennia and, is striving to live on in the face adversities including those resulting from Climate Change and anthropogenic alterations.

Marine microbes flourish from the top millimeter of the ocean surface to several kilometers below the seafloor. A few microliter of seawater contains more bacteria than the people on this planet. In addition to being copious, the numerous different types of marine bacteria carry out many different types of metabolism, supplying oxygen, are the major processors of the world's greenhouse gases and have the potential to ease the effects of climate change. The metabolic processes that they carry out in the transformation of elements,

degradation of organic matter, and recycling of nutrients play a central role in innumerable activities that affect the support and maintenance of all other forms of life.

Oxygen is the favored electron acceptor for the respiration of organic matter allowing marine bacteria to lead an oxic mode of life. Deoxygenation of Marine Ecosystems in the recent past is an example of Changing Oceans. Deoxygenation is loss or reduction of dissolved oxygen consequentially leading to hypoxia, suboxia and anoxia. However, in certain parts of the ocean such as the eastern tropical North Pacific (ETNP) and South Pacific (ETSP) in the Pacific Ocean, the Arabian Sea and the Bay of Bengal in the Indian Ocean, the eastern tropical South Atlantic (ETSA) in the Atlantic Ocean, oxygen drops to 4.5 μmol kg^{-1} such that oxic respiration can hardly be sustained (Naqvi et al. 2000; Karstensen et al. 2008; Keeling et al. 2010). Even though most life avoids such low oxygen conditions, those bacteria that can exploit alternative electron acceptors for respiration thrive in these suboxic waters forming unique communities distinct from those living in oxic waters.

Nitrate (NO_3^-) is the next preferred electron acceptor in the electrochemical series of reductants, for respiration after oxygen and can yield similar amounts of free energy as that from oxic respiration of organic matter (Froelich et al. 1979). The expected energy yield of the reaction $NO_3 \rightarrow N_2$ is followed by manganese and iodate reduction, while the energy yield from iron and sulfate respiration is significantly lower (Lam and Kupers, 2011). Nitrate and iodate occur at relatively high concentrations (~30 μM and 0.2–0.5 μM, respectively) in typical seawater, compared with only (sub) nanomolar levels of both manganese and iron. Therefore, nitrate reduction is significant in suboxic respiration in seawater.

Marine nitrogen cycle is possibly the most complex and the most interesting among all the biogeochemical cycles in the sea. Nitrogen is the limiting element for biological

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productivity, occupying a central role in ocean biogeochemistry; nitrogen exerts significant influence on biogeochemical cycles of many other elements. Nitrogen with a myriad of unique chemical transformations in various oxidation states, ranging from -3 (ammonium and amino-nitrogen) to $+5$ (nitrate) is a vital element for biological functioning. All these transformations are mediated by marine-organisms as part of their metabolism, either to gain energy for their growth or to synthesize structural components viz, peptides, amino acids, enzymes and structural proteins.

With the advent of molecular tools in microbial ecology, it's easier to study bacterial community composition and obtain essential information on the significance of specific species/genus/groups of bacteria. The discovery that these tiny microbes are present large numbers, and are largely responsible for the biogeochemical processes that shape our planet can be viewed as one of the most important advances in science.

The principle of ecosystem ecology is to understand how the ecosystems maintain functional stability and predicting how ecosystems respond to environmental changes (Rastogi et al. 2011). In any ecosystem interacting biological entities with their physical environment, three fundamental questions that arise while discovering and characterizing any ecosystem are:

(1) What type of microorganisms are present?

(2) What do these microorganisms do?

(3) How are the activities of these microorganisms related to ecosystem functions (e.g., energy flow, biogeochemical cycling, and ecological resilience?)

Marine microbial ecology aims to answer these questions and deals with the study of microorganisms, their interactions with each other and within their marine environment. It is

an emerging field of science as the abundance and diversity of marine microbes are now appreciated as essential in mediating various biochemical cycles and other processes that influence the Earth's climate. Understanding how microbes adapt rapidly to changes in their external environment with continued high growth rates is one of the major research challenges. In this regard, deoxygenation (loss or reduction of dissolved oxygen) of marine ecosystems in the recent past is an example of Changing Oceans. Deoxygenation is consequentially leading to hypoxia, suboxia and anoxia. Owing to their resilience to thrive, the microbial communities extant in the low oxic marine zones are of vital biogeochemical and ecological significance. Exploring the mechanisms underlying their diversity and their role in ecosystem functioning will continue to be of pertinence, times to come.

Marine microbiology focuses majorly to recognize how bacterial assemblages vary in space and time along environmental gradients, among geographic locations and among time-scales. The present study deals with bacterial communities from the coastal ecosystem in response to changing the environment, in particular to the seasonal intensification of coastal hypoxia. For this, three geographically separated coastal environs off marine water columns: Goa, Mangalore, and Kochi along the west coast of India were chosen. These locations are characterized by seasonal upwelling conditions leading to hypoxia. Therefore, it was hypothesized that the bacterial diversity in these locations ought to respond to temporal changes as well as to reduced oxygen concentrations. This study also aimed at identifying the phylogenetic differences of bacterial communities between different periods of the year from these locations.

The following objectives were outlined for this study

❖ To analyze the spatiotemporal variations in bacterial communities by culture-dependent and culture-independent approach in the west coast of India.

The basis of this objective was to introduce the various types of bacterial assemblages found in this marine environment, i.e., "Who is there?". Describing the bacterial community structure, we can deduce what they could be doing. Spatiotemporal variability was conceived with the idea to realize the community structure-differences/similarities within the west coast which experience seasonal variability in its physical and chemical settings. Therefore, to obtain a complete idea of the extant bacterial community, the study resorted to having an idea of culture amenable bacteria as well as to clone and sequence the 16S rRNA gene amplicons as to obtain information on the operational taxonomic units of Bacteria.

❖ Through metagenomic analyses document the spatiotemporal variations of nitrogen metabolizing phylogenetic assemblages along the west coast of India. Molecular screening for functional gene fragments in metagenomic DNA helps to understand the ecological functions of bacteria under adverse conditions in response to the changing environmental conditions. This would answer "What do they do?". Sequencing and annotation of functional gene markers are useful for describing the hitherto uncharacterized majority of environmental microorganisms. Besides aiding in the detection of new microbial lineages and these approaches are well known for enabling the description of genetic and functional diversity.

❖ To elucidate the role of certain culturable strain/s in utilization and transformation of N species.

To comprehend the physiology and ecology of bacterial species, their isolation in pure culture remains an essential step in microbial ecology. Culture-based studies provide information on the physiological characteristics of the organisms and their activity to the functioning of the ecosystem.

REVIEW OF LITERATURE

2.1. <u>Introduction</u>

Coastal oceans within 200 m isobaths and covering 7% of the total oceanic area are variously important. Being biologically highly productive compared to any other part of the ocean, they are more directly affected by human activity. Located between the land and the main sea, the coastal ocean plays a significant role in biogeochemical cycling; exchanges energy and matter with Open Ocean, assimilates or accumulates terrestrial inputs of freshwater, dissolved/particulate nutrients, sediments, and organic matter. Even the groundwater flow and surface runoff have to pass through it (Gattuso et al. 1998; Gattuso and Smith, 2007). Close to 90% of the world fish catch is supported by this swath of the ocean. As much as half of the world population lives within 100 km of the coastlines, making the coastal ocean extremely vulnerable to anthropogenic impingement and deterioration. Thus, coastal ecosystems are facing several perturbations including losing the dissolved oxygen concentration what the modern chemists term severally as coastal hypoxia, suboxia or deoxygenation.

2.1.1 <u>The coastal hypoxia</u>

Oxygen is the preferred electron acceptor for the respiration of organic matter in the marine environment enabling marine organisms to lead an oxic mode of life. However, certain regions of the marine ecosystem experience shortage of oxygen called as hypoxia. Marine hypoxic regions are the regions of the world in which the dissolved oxygen of the sub-surface waters fall to near zero levels (≤ 1.4 ml L^{-1}); due to the high demand of oxygen in degradation of organic matter by microbes, caused either human-induced (anthropogenic activities) or due to natural events like upwelling (Diaz and Rosenberg, 2008; Levin et al. 2009). Such regions

are hotspots forcing the habitual microbes to utilize an alternate mode of electron acceptors, preferably nitrate for their survival and making a significant contribution to the global cycling of nitrogen through denitrification. Thus, such oxygen-deficient regions being a medium for the biochemical transformation of nitrogen species, i.e., nitrate exerts a direct control on the climate through nitrous oxide produced (Codispoti and Christensen, 1885).

2.1.2 Global scenario of coastal hypoxia

The oxygen depletion is known occur to naturally along the coastal regions of Atlantic and Pacific oceans, in response to upwelling. Upwelling involves wind-driven motion of dense, cooler and usually nutrient-rich water towards the ocean surface replacing the warmer usually nutrient-depleted water, resulting in intense biological productivity and the higher oxygen demand for degradation of organic matter leads to oxygen depletion. Hypoxia develops at irregular intervals greater than a year (aperiodic); at regular intervals shorter than a year (periodic); yearly due to summer or autumnal stratification (seasonal) or year-round hypoxia (persistent) (Diaz, 2001). The coastal hypoxic system globally is listed in Table 1.

Ocean indeed is a large source of potent greenhouse gas nitrous oxide (N_2O) to the atmosphere, accounting for a one-third of all emissions (Bange, 2007). Because of dentrification beginning from nitrate reduction, formation of different gaseous species of nitrogen including nitrous oxide, the potent greenhouse gas is through catabolic processes of certain microbes. On a per molecule basis, nitrous oxide is ~25 times more potent than carbon dioxide (CO_2) in trapping heat, which influences the Earth's climate. In the troposphere it traps the heat and, in the stratosphere (Montzka et al. 2003; Prather et al. 2015), it is involved in affecting the ozone cycling.

Off Southwest Africa (Eastern Atlantic Ocean)

The highest primary productivity is supported by upwelling off Nambia along Southwest Africa) in the south eastern Atlantic Ocean (Carr, 2002), and respiration of the abundant organic matter produced in the water column and shelf sediments terminates in anoxia. Sulphidic condition is observed in the near bottom layers extending offshore to the shelf off the Walvis Bay (Br¨uchert et al. 2006, 2009). Off Cape Frio in the north and off Luderitz in the south are the two main upwelling sites in this region. The intermediate water is upwelled off Cape Frio and off Luderitz by the hypoxic Angola Basin Central Water and oxygenated Cape Basin South Atlantic Central Water respectively. The seasonal plus inter-annual variability of oxygen deficiency at these sites is regulated by the movement of these waters northward from Luderitz or southward from Cape Frio (Monteiro et al. 2008). Kuypers et al. (2005) reports the significance of this region for redox nitrogen transformations.

Eastern Tropical North Pacific

The surface current (California Current) flow equatorward at the same time as the northwesterly winds cause intense upwelling along the west coast of the United States and the northwest coast of Mexico. Low oxygen waters < 0.2 ml L^{-1} extends offshore from the coast and the area is reported for nitrogen cycling (Cline and Richards, 1972).

Eastern Tropical South Pacific

The surface Humboldt Current carries low-salinity and oxygenated Subantarctic Water equatorward and, the subsurface Peru-Chile Undercurrent transports high nutrient, high-salinity, and low-oxygen Equatorial Subsurface Water poleward. Upwelling along the

Peruvian Chilean coast brings cold, nutrient-rich, oxygen-poor waters to the surface. The subsequent high biological productivity is responsible for the intense oxygen depletion worldwide that extends from very shallow depths far offshore into the open ocean. Oxygen deficiency, complete denitrification and associated sulphidic conditions occur near Peru, and Chile is reported to observe oxygen depletion during spring and summer (Dugdale 1977; Lam et al. 2009, Paulmier 2006).

European coastal water hypoxia

The major seasonally-occurring hypoxia has been reported from the coastal Caspian Sea (Djakovac et al. 2015) Adriatic Sea (Justić, 1991; Druon et al. 2004), Baltic Sea (Conley et al. 2011), Black Sea north western shelf (Daskalov, 2003).

Coastal hypoxic zones formed due to anthropogenic activities are mainly located in nearshore waters and estuaries that receive copious discharge of nutrients or organic matter through river runoff. In addition to this, the freshwater stratification makes these regions vulnerable to oxygen depletion. The coastal region off Changjiang and Mississippi rivers located in the East China Sea and the Gulf of Mexico respectively, the Chesapeake Bay and Tokyo Bay are few examples of hypoxic regions formed due to human-induced activities. Deoxygenation in such systems depends on the solar radiation as well as changes in river runoff and is known to maximize in summer (Rabalais and Turner, 2006; Zhang et al. 2008, Kodama et al. 2011).

Table 2.1: The global coastal hypoxic regions (*Modified from Diaz, 2001, Kim et al.* 2005a, *Lee et al. 2018*)

Loch Creran, Scotland	Persistent
Byfjord, Sweden	
ldefjord, Sweden—Norway	
Baltic Sea, Central	
Fosa de Cariaco, Venezuela	
Gulf of Finland	
Black Sea(except North Western shelf)	
Caspian Sea	
New York Bight, New Jersey	Aperiodic
Shallow Texas Shelf	
Deep Texas Shelf	
German Bight, North Sea	
Sommone Bay, France	
North Sea, W. Denmark	
York River, Virginia	Periodic
Rappahannock River, Virginia	
Chenosu Bay, West Coast of Korea	
Long Island Sound, New York	Seasonal
Main Chesapeake Bay, Maryland	
Pamlico River, North Carolina	
Mobile Bay, Alabama	
Hillsborough Bay, Florida	
Louisiana Shelf	
Bomholm Basin, S. Baltic	
Oslolord, Norway	
Kattegat, Sweden—Denmark	
German Bight, North Sea	
Laholm Bay, Sweden	
Gullmars fjord, Sweden	
Swedish west coast fjords, Sweden	
Limqord, Denmark	
Kiel Bay, Germany	
Lough Ina, Scotland	
Gulf of Trieste, Adriatic	
Elefsis Bay, Aegean Sea	
Arhus Bay, Denmark	
Seto Inland Sea, Japan	
Saanich Inlet, British Columbia	
Port Hacking, Australia	
Tolo Harbor, Hong Kong	
Japan, all major harbors, Japan	
Tome Cove, Japan	

Black Sea, North Western Shelf	

Table 2.1 contd...

Gulf of Mexico	Seasonal
Jinhae Bay, Southern Coast, South Korea	
Gamak Bay, Southern Coast, South Korea	

2.2 <u>Bacterial community composition in oxygen deficient regions</u>

As has been understood these days, in all of the marine ecotypes, bacteria are of great ecological importance playing many vital roles in the cycling of most biologically essential elements (Azam et al. 1994) and many other processes in the marine ecosystem. Their role in the structuring of marine trophic web networks and, remineralization of organic matter (Azam et al. 1983; Azam and Long, 2001; Ducklow et al. 2002) is paramount. Therefore it is a requisite to study the phylogenetic groups of bacterial communities dominating in the marine environment as the abundant groups may have different roles in biogeochemical processes (Cottrell and Kirchman, 2000). Though most life does not tolerate low oxygen conditions, those microbes that can exploit alternative electron acceptors to support respiration, flourish in low oxygenated waters and form unique microbial communities distinct from those living in oxic waters. Several authors have reported bacterial community from low oxygen marine environments worldwide. Wright et al. (2012) reviewed the bacterial community composition in open-ocean and coastal oxygen deficient zones and enclosed or semi-enclosed euxinic basins including the Northeast subarctic Pacific (NESAP), the eastern tropical South Pacific (ETSP), the Namibian upwelling, and Saanich Inlet, based on taxonomic surveys of 16S rRNA gene sequences. Major groups found in order of abundance included *Proteobacteria*, *Bacteroidetes*, *candidate division Marine Group A*, *Actinobacteria*, *Planctomycetes*,

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Cyanobacteria, *Firmicutes*, *Verrucomicrobia*, *Gemmatimonadetes*, *Lentisphaerae*, *Chloroflexi* and some candidate divisions. Ye et al. (2016) reported the presence of *Gammaproteobacteria*, *Bacteroidetes,* and *Planctomycetes* from a hypoxic zone in the East China Sea. Investigations in the Arabian Sea from the oxygen minimum zone sediment and water column by Divya et al. (2010) and Jain et al. (2014) reported the presence of *Gammaproteobacteria*, *Alphaproteobacteria*, *Betaproteobacteria, Deltaproteobacteria*, *Planctomycetes*, *Verrucomicrobia, Spirochetes,* and *Bacteroidetes*. These recurring bacterial community compositions within the oxygen deficient zones stress the significance of oxygen concentration as an organizing principle in pelagic bacterial communities.

Due to ongoing changes in oxygen distribution all over the world oceans that include the appearance of numerous hypoxic zones due to eutrophication, and/or, in some cases, changes in circulation (Diaz and Rosenberg, 2008; Stramma et al. 2008) such environments have drawn attention to study the key players involved in regulating the stability of the ecosystem in response to changing environmental conditions. The high abundance of bacteria inhabiting such ecosystems are capable of degrading the organic matter, multiple functions in the nitrogen and other elemental cycles. Their easy adaptability to changing environmental conditions versus actual activities presents a challenge to ecophysiological and biogeochemical measurements.

2.3 The Process of Marine Denitrification

Denitrification is the important component of nitrogen cycling in the west coast of India. Nitrogen being a polyvalent element, its transformation is greatly affected by the surrounding oxygen concentration in the marine environment. As seawater is generally

oxygenated, fixed nitrogen in the ocean largely ends up in the most oxidized (+5) state, viz. nitrate ions (NO_3^-), and only in sediments and in parts of the water column in a few well-demarcated regions like the west coast of India, the dissolved O_2 gets almost completely depleted to allow microbial conversion of NO_3^- to molecular nitrogen (N_2); this process, denitrification, is the most important pathway of losses of fixed nitrogen.

2.3.1 Factors governing denitrification

Denitrification refers to the microbially mediated sequential reduction of soluble nitrogen forms nitrate (NO_3^-) and nirite (NO_2^-) to the gaseous products nitrous oxide (N_2O) and/or nitrogen (N_2) via nitrite and nitric oxide termed as heterotrophic denitrification, as organic matter is respired in the process, the water-soluble nitrate is converted into gaseous nitrogen containing gases. These are

    (i)   The cytotoxic and ozone-depleting nitric oxide (NO),

    (ii)   Potent and long-lived greenhouse gas nitrous oxide (N_2O) and

    (iii)   The relatively inert dinitrogen gas (N_2).

It is a facultative anaerobic and one of the most important processes in the nitrogen cycle as it refluxes back to the atmosphere some of the N_2 added to the ocean. Almost all heterotrophic denitrifiers are facultative anaerobes (Tiedje, 1989). Normally, denitrification occurs when these conditions are satisfied:

i) Nitrogen sources for denitrification are available

ii) Oxygen concentrations are reduced

iii) Electron donors are available (Seitzinger et al. 2006).

Denitrification requires an input of electron acceptors (oxidized N) from advection (presumably deep, nitrate-rich water) in suboxic waters. Upwelling centered around May to October along the west coast of India induces nutrient-rich waters from the bottom to the surface. The upwelled water reaches the surface layer in May and intensifies during June–July but withdraws completely and abruptly by October. Initially, the region is hypoxic ($\leq$ 1.4 ml L^{-1}); however, as it moves up the shelf, it loses O_2 due to the degradation of organic matter sinking from productive surface waters. As the O_2 levels decline fall below a value of ~1–5 μM (0.02–0.11 mL L^{-1}), there occurs a shift to what is often referred to as the suboxic ecosystem. Under these conditions, facultative heterotrophs switch over to alternate respiratory oxidants (electron acceptors) (Bange et al. 2005; Jayakumar et al. 2009a, Gupta et al. 2016).

Owing to the oxidizing nature of the Earth's surface environment, including most of the oceanic water column, nitrate (NO_3^-) is undoubtedly the most abundant combined nitrogen species in aquatic systems and occurs at a relatively high concentration (30 μM) in seawater. Nitrate (NO_3^-) being the next favored electron acceptor for respiration after oxygen (considering the reduction to dinitrogen, N_2) and can yield similar amounts of free energy as that from oxic respiration of organic matter (Froelich et al. 1979, Bange, 2005). Denitrification occurs where nitrate is present in congruence to reduced oxygen concentrations. This occurs in specific habitats, such as the oxic-anoxic interface of benthic sediments, and in the water column at the edge of hypoxic, suboxic or anoxic water masses in Oxygen Minimum Zones and upwelling regions. The poor availability of oxygen in the water column forces the microbes to utilize nitrate as the next terminal electron acceptor to support respiration resulting in denitrification.

2.3.2 Bacterial mediated denitrification and genes involved

Denitrification constitutes one of the main branches of the global nitrogen cycle sustained by bacteria, as it uses NO_3^-, NO_2^-, and nitrogen oxides (NO, N_2O) as terminal electron acceptors to support respiration in low concentration/absence of oxygen. The process involves four enzymatically catalyzed reaction steps: nitrate reduction, nitrite reduction, nitric oxide reduction, and nitrous oxide reduction (Knowles, 1982). The end product is generally N_2, although a few bacterial species terminate at N_2O production (Payne, 1981). The genes involved in denitrification are indicated by italics.
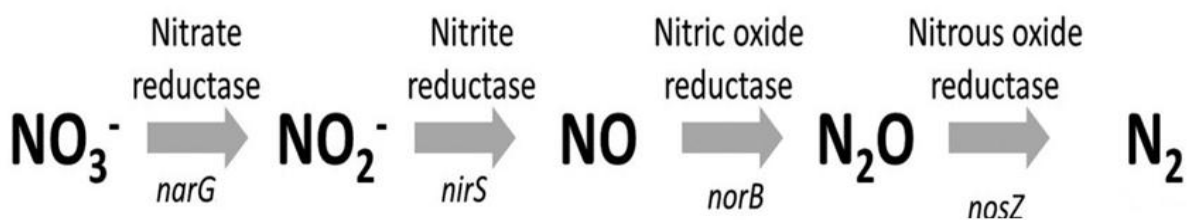
(Modified from Alvarez et al. 2014)

The ability to denitrify has been identified among bacteria, fungi, and archaea. However, fungal and archaeal denitrification is not the focus of this research. A very diverse group of phylogenetically unrelated bacteria, including members of the *Aquificae*, *Deinococcus-Thermus*, *Bacteroidetes*, *Firmicutes* (*Bacillus* sp.), *Actinobacteria* (*Streptomyces* sp.) and *Proteobacteria* (*Pseudomonas*, *Ralstonia*, *Alcaligenes*, *Paracoccus*, *Rhodobacter*, *Marinobacter*, *Rubrivivax, Thauera, Burkholderia* sp.) are known to have the ability to denitrify (Shoun et al. 1992; Zumft 1997; Philippot 2002).

Denitrification is considered to be a community process, as many denitrifying organisms do not possess the complete set of enzymes to complete the reaction, and could, therefore, work together to complete the process (Zumft 1997). Taxonomically diverse groups of bacteria are capable of reducing nitrate to nitrite carried out by two types of enzymes, membrane-bound and periplasmic-bound nitrate reductases encoded by *nar* and *nap* genes respectively. *narG* is expressed predominately under anaerobic denitrifying conditions, and *napA* under aerobic conditions (Bell et al. 1990). Nitrite reduction is carried out by two types of the nitrite reductases (*nir*), either a copper-containing enzyme encoded by *nirK* or a cytochrome cd1 encoded by *nirS* gene. A bacterium can have one of the two nitrite reductases, but not both. As reported by Jayakumar et al. (2009a), the *nirS* gene is found nearly exclusively in *Proteobacteria*, but Strous et al. (2006) reported this gene from *Planctomycetes,* while *nirK* gene has been reported from many diverse taxa (Zumft, 1997). The reduction of nitrogen oxides, i.e., NO, N_2O are carried out by *norB* and *nosZ* genes respectively. The *nosZ* gene, encoding for nitrous oxide reductase, it regulates the consumption of N_2O. The reduction of N_2O to N_2 by this enzyme is the only known mechanism to remove N_2O from the atmosphere, other than photolysis and oxidative reactions in the stratosphere (Montzka et al. 2011).

Over 60 genera of bacteria, widespread across many taxonomic groups can denitrify but rarely are they strict anaerobes (Zumft, 1997). In fact, most of them can switch between oxic and NO_3^- dependent modes of respiration. The transcription and subsequent build-up of denitrifying enzymes occur when their mode switch, which is mainly regulated by oxygen and NO_3^- availability. Each step in denitrification is mediated by different enzymes. The transcription and activities of each enzyme respond differently to oxygen, NO_3^-, NO_2^-, NO

and N_2O and their activities are not necessarily coordinated with considerable interspecies variations (Ferguson, 1994). For example, except for the reaction of membrane-bound nitrate reductase (*nar*) that occurs in the cytoplasm, enzymatic reactions of the next three steps of denitrification all take place in the periplasm, which means that, following its production, NO_2^- must be transported across the membrane back to the periplasm for subsequent reduction (Zumft, 1997).

As nitrous oxide (NO) is a free radical and, thus, very reactive, and highly toxic to most bacteria including denitrifiers (Zumft, 1997), nitrite reductase and nitric oxide reductase are controlled interdependently at both the transcriptional and enzyme activity levels in order to minimize the accumulation of NO (Ferguson, 1994; Zumft, 1997). Although the distribution of NO in the environment has not been well-studied, it is generally undetectable in aerobic water columns, but it has been detected at low levels (≤ 0.5 nM) in oxygen deficient waters (Ward and Zafario, 1988).

2.3.3. Molecular markers for characterizing denitrifying bacteria

The advancement in molecular microbiology has swept through the various fields of marine microbial ecology. Studies on diversity and distribution of marine phylotypes and the distribution of specific functional genes are now a large component of research in this field. The target functional genes are amplified using PCR; where the primers are specifically designed to target the gene of interest based on sequence alignments found in the GenBank databases. For each denitrification gene, there are very few full-length sequences, as these are typically obtained from genome sequencing of cultured microbes. Therefore, the extent to which these primers target all variants of these genes is difficult to assess.

The physiological trait such as denitrification is not limited to specific microbial taxa and is therefore studied independently of culture through the relevant functional genes designed for the same. The genes encoding the catalytic subunits have extensively been used as signature genes to characterize the composition of denitrifier communities in past decade and have been extensively studied in various environments such as sludge, soils, sediments, etc. The ability to denitrify at irregular intervals is distributed both within and between different genera and cannot be associated with any specific taxonomic group. Therefore, existing techniques to study the ecology of denitrifiers are based on the use of the functional genes in the denitrification pathway or their transcripts as molecular markers of this community. DNA extraction followed by PCR amplification of denitrification genes has been the most common way to start-off the analysis of denitrifier communities.

Over the past decades, primer pairs and PCR protocols have been developed for the amplification of *narG*, *nirS* and *nosZ* genes to describe denitrifier communities (Braker et al. 1998; Gregory et al. 2000; Bru et al. 2007; Lee at al. 2009; Zhang et al. 2016). Amplifications directly from environmental DNA tend to reveal a greater degree of sequence diversity in nitrite and nitrous oxide reductase genes than is apparent in the same genes from cultured isolates (Braker et al. 2000; Scala and Kerkhof, 1998).

Of the two nitrate reductases (membrane and periplasmic), the membrane-associated enzyme (*narG*) is typically involved with nitrate respiration under hypoxic conditions and probably has a greater role to play in the environmental nitrogen cycle (Richardson et al. 2001). Hence, this study has focused on the membrane-associated nitrate reductase and makes use of previously developed PCR primer systems that successfully amplify fragments of the

narG gene that encodes the catalytic molybdenum-cofactor-containing subunit of the enzyme. Taxonomically diverse group of bacteria can reduce nitrate to nitrite.

Genes coding for nitrite reductase (*nir*) were the first to be used for studies of denitrifier diversity and have subsequently been the most common molecular marker for denitrifier community studies (Braker et al. 1998; Jayakumar et al. 2004). These genes were used as nitrite reduction is the first step in the denitrification reaction that results in the production of a gaseous product. Braker et al. (2000, 2001), Liu et al. (2003) and Scala et al. (1999) explored marine denitrifier communities from sediments and Castro-González et al. (2005), Hannig et al. (2006) reported at oxic–anoxic interfaces in the water column. Sequence identity levels as low as 45% (Braker et al. 2000), were detected from marine sediments with *nirS* gene primers.

Not many studies have focused on nitric oxide reductase (*nor*) using *norB* as a gene target (Braker and Tiedje, 2003). Nitrous oxide reductase (*nosZ*) has been quite extensively used to describe denitrifier communities because this enzyme reduces a potent greenhouse gas N_2O, to N_2 which is relatively inert in the atmosphere. The application of extraction of DNA from environmental matrices in combination with PCR routed the development of culture-independent approaches in microbial ecology. Since the early 1990s (Giovannoni et al. 1990), these methods, have been applied allowing the total microbial community analysis present within environmental systems, have revolutionized our understanding of microbial community structure and diversity within the environment.

Quantitative-PCR or qPCR is now widely used in microbial ecology to determine gene numbers present within environmental samples, allowing quantification of functional gene markers present within a community from the domain level down to the quantification of

phylotypes or individual species. It is the highly reproducible, robust and sensitive method to quantitatively track phylogenetic and functional gene changes across spatiotemporal scales under varying environmental conditions. The quantitative data obtained can be used to relate disparity or variation in gene abundances in comparison with variation in biotic or abiotic factors and/or biological activities and process rates (Smith and Osborn, 2009). The abundance of denitrification genes by qPCR encoding the nitrate, nitrite, and nitrous oxide reductases have been used to study denitrifiers from volcanic soils, glacier foreland, wastewater, deep sea sediments etc (Chon et al. 2011; Li et al. 2013; Wyman et al. 2013; Yu et al. 2014; Yi et al. 2015; Carvajal et al. 2016).

2.4. Exploring bacterial diversity by 16SrRNA gene

The great plate count anomaly coined by Stanley and Konopka (1985), that is, the variability in bacterial abundance between the number of cells that could be grown as colonies on agar plates and the number of bacterial cells enumerated in seawater by epifluorescence microscopy, concluded that a very large portion of bacteria cannot be currently grown under laboratory conditions, some are non-viable, while some may be viable but non-culturable. The bacterial isolates that were obtained for use in laboratory experimentation were typically microbes that grew well at high substrate concentration. Microbiological culture techniques have been significantly improved ever since the discovery of bacterial pure culture techniques by Robert Koch. Traditionally bacteria were identified based on its morphological and physiological properties using Bergey's manual systems of classification based on morphology, Gram stain, spore stain, motility, biochemical characterization, enzyme activities, and utilization of several substrates as sole carbon and energy sources which

allowed the grouping of bacterial isolates into genera and species. These approaches have been used to identify and characterize the culturable bacteria. However, a majority of bacterial species in any environment are still uncultivable in the laboratory, due to the lack of knowledge of the real conditions under which these bacteria are growing in their natural environment. Because of this limitation, bacterial diversity can only be accurately determined using molecular taxonomic tools that preclude the need for laboratory cultivation.

The use of smaller subunit ribosomal RNA (rRNA) sequencing established by Carl Woese in the 1970s provided the foundation for determining the evolutionary relationship between organisms and thereby quantifying diversity as sequences-divergence on a phylogenetic tree, improved the view of microbial diversity. Norman Pace and colleagues (Pace et al. 1986; Olson et al. 1986) proposed the outline of a molecular approach to identify the 16S rRNA gene (16S rDNA) of microbes without their cultivation. In this method, DNA is extracted from environmental samples and the 16S rRNA gene amplified via PCR (using universal primers for 16S rDNA), and PCR amplicons were cloned into competent cells and subjected to DNA sequencing. The 16S rDNA sequences obtained are then compared with sequences in the NCBI database using Basic Local Alignment Search Tool (BLAST). These sequences are then compared with known sequences to find their position in a phylogenetic tree.

Microbiologists have primarily depended on 16S rRNA gene sequencing for identification and classification of isolated pure cultures and 16S rDNA-based metagenomic analysis of bacterial diversity (Stevens and Ulloa, 2008; Alonso-Gutiérrez et al. 2009; Cury et al. 2011; Divya et al. 2011; Da' Silva et al. 2013; Ye et al. 2016). This gene has several conserved regions which are common to a large number of bacterial species, and variable

regions, which are shared by fewer species. Primers are designed to bind the conserved regions and amplify variable regions. The 16S rRNA gene sequences used to study taxonomy and bacterial phylogeny has been by far the most common genetic marker used for some reasons that include:

I. Its presence in almost all bacteria they contain variable and highly conserved regions which allow distinguishing between organisms on all phylogenetic levels

II. The 16S rRNA gene over time is the same and has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution);

III. A lot of data exist in the databases such as BLAST (http://www.ncbi.nlm.nih.gov) which can be used to compare the DNA-sequences of unknown microorganisms and allow a phylogenetic identification. Species are identified based on the closest match obtained from the existing database.

Because of the significance of bacteria in the marine environment, it is important to understand the full extent of bacterial diversity and the role of the most abundant species. Comparisons between classical culture-dependent and 16S rDNA-based metagenomic analysis of bacterial diversity have revealed that only about 1% of the total microbes are amenable to culture. To comprehend the physiology and ecology of bacterial species, their isolation in pure culture remains an essential step in microbial ecology. Using both 16S rDNA phylogenetic analysis and culture techniques, it is possible to characterize the microbial

diversity and culture characteristics of the isolated microorganisms in different environments, allowing a complete picture.

2.4.1. Phylogenetic and statistical analyses of bacterial diversity

The culture-independent studies of the microbial diversity are mostly based on 16S rDNA/functional gene sequences. A number of statistical models are used to compare the bacterial community richness that depends on the operational taxonomic units (OTUs). OTUs are a cluster of similar variants of the 16S rRNA/functional marker gene sequences. Each of these clusters represents a taxonomic unit of bacterial genus depending on the sequence similarity threshold. Usually, OTU cluster is defined by a 97 % identity threshold of the desired gene sequence variants at the genus level. From each OTU, a single sequence is selected as a representative. This representative sequence is annotated, and that annotation is applied to all remaining sequences within that OTU.

Once OTUs are defined it is possible to compare and estimate species richness. A commonly applied procedure is the rarefaction analysis, which compares observed richness in environments that have not been sampled equally (Hughes et al. 2001). In any sampling regimen, the number of types increases with the effort until all types are observed. This relationship allows accumulation or rank abundance curves to be plotted, the shape of which provides information on how well the community has been sampled relative to its total diversity (Kemp and Aller, 2004).

The nonparametric species richness estimator Chao 1 (Chao, 1987) and Goods's coverage (Good, 1953) has been applied to microbial data sets in ecological studies. Species

richness estimators are used to estimate the total number of species present in a community. The *Chao 1* index is commonly used and is based upon the number of rare classes (i.e., OTUs) found in a sample (Chao, 1984). Goods coverage estimates the percentage of the total species represented in a sample. The combination of species richness and diversity estimates provide information that enables a deeper understanding of microbial diversity. The software program MOTHUR is very helpful for the statistical analysis of microbial diversity (Schloss et al. 2009).

2.5 <u>West coast of India: Studies on its circulation pattern and Biogeochemistry</u>

The contribution of the Indian west coast to biogeochemical cycling of nitrogen is disproportionately large. It is one of the major sites where fixed nitrogen, i.e., nitrate, is transformed in the water column to dissolved gaseous nitrogen (Bange et al. 2005). This process is called pelagic denitrification (to distinguish it from sedimentary denitrification). The Arabian Sea accounts for 15% of oceanic primary production (Prasannakumar et al. 1995), 80% of organic burial (Paropkari et al. 1992), 50% of calcium carbonate deposition (Naidu, 1991; Paropkari et al. 1992; Pattan et al. 2003), 90% of sedimentary mineralization (Nair et al. 1989), and 75–90% of oceanic sink of suspended material carried by rivers.

2.5.1. Circulation pattern along west coast of India

In coastal regions, winds and currents are the primary driving forces which determine the physical processes of the system. The co-existence of physical processes and their interaction with each other has a significant influence on ecology and coastal geomorphology. In the west coast of India, strong winds blowing parallel to the coast during the southwest monsoon (June-October), force the surface waters embracing the coastal regions to move

away from the shore resulting in a net movement of surface water at right angles to the direction of the wind (45º at surface to total shift of 90° for the water column), which is termed as Ekman transport (Rao et al. 2008). When Ekman transport occurs along the coast, the surface waters are replaced by drawing those from intermediate depths to surface, this process is known as upwelling (Fig 1). In the reverse process, Ekman transport moves the surface waters toward the coast, which piles up and eventually sinks, this process referred to as downwelling (Fig 2) occurs during the northeast monsoon (November to March).

Deep waters are enriched with nutrients such as nitrate, silicate, and phosphate due to the decomposition of sinking organic matter and lack of biological uptake. These nutrients when brought to the surface results in intense biological productivity. Coastal upwelling systems are highly active and exhibit wide variations in the hydrographic, nutrient and phytoplankton characteristics controlled by remote forcing on longer timescales and local meteorology on short timescales. Upwelling regions in comparison to other areas of the ocean are therefore significant for very high levels of primary production. Though the upwelling phenomenon is less in intensity along the south west coast of India in comparison to the other thoroughly studied upwelling regimes of the Arabian Sea (like those at Somalia and Oman), it has profound impacts on the coastal fisheries of India. While the west coast of India accounts for 70 % fish yield of the total Arabian Sea production, the southwest coast alone accounts for 53 % hence this region is of considerable importance in the Indian context (Priya et al. 2016).
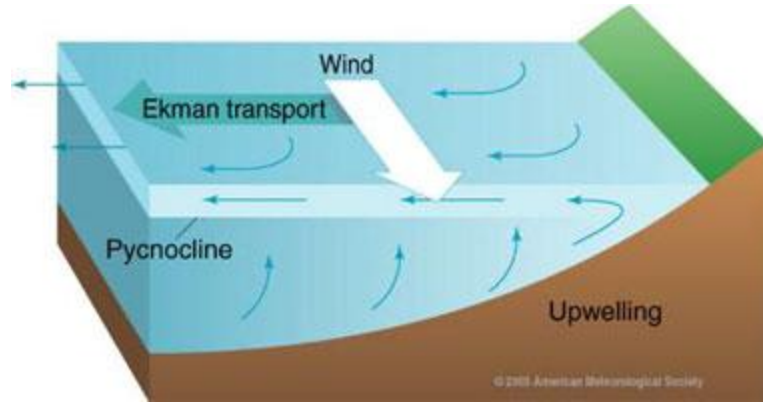
Fig 1: Upwelling
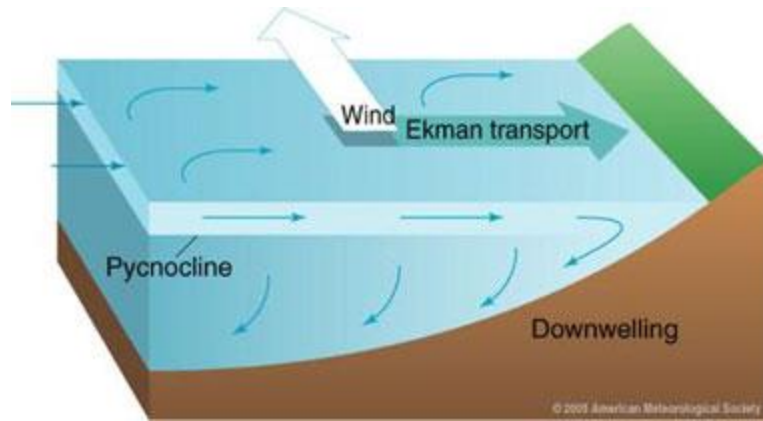
Fig 2: Downwelling

(Adapted from DataStreme Ocean)

2.5.2. The evolution of seasonal hypoxia along the west coast of India

The existence of permanent hypoxia in the Indian Ocean (Arabian Sea) was discovered during the first major scientific expedition—the John Murray Expedition in 1933–1934. The thickest oxygen (O_2) deficient zone in the open-ocean was first discovered (Sewell and Fage, 1948) and thereby nitrogen cycling investigated (Gilson, 1937). Sen Gupta et al. (1976) investigated nitrogen cycle processes in the region. Since then a lot of research has been conducted on the northwestern Indian Ocean. In addition to the oxygen deficient zone in the open-ocean, the Arabian Sea, on the other hand, houses one of the major upwelling zones of the world: the west coast of India, during the southwest monsoon (June-October) (Banse, 1959; Carruthers et al. 1959). The first time series measurement was made by Banse off Kochi (latitude 10^oN) during 1958–1960 (Banse, 1959; 1968) observing the decline of oxygen in near-bottom waters during April as the water column began to get stratified with the peak oxygen depletion occurring during September–October. A similar pattern was also seen in waters off Goa at a fixed regular sampling site the Candolim Time Series (CaTS) since 1997 (Naqvi et al. 2000, 2006).

The surface currents in the Arabian Sea flow clockwise during the southwest monsoon and anti-clockwise during the northeast monsoon (Naqvi, 2008). The temporal evolution of hypoxic conditions over the west coast of India is linked to the seasonal reversal of surface circulation; the southward or clockwise flowing West India CoastalCurrent (WICC) that induces upwelling. The oxygen deficiency begins with the initiation of upwelling, sometimes

April–May and intensifies gradually with time (Naqvi, 2006; Naqvi and Unnikrishnan, 2009). The intense biological productivity as a result of upwelling results in a greater demand for oxygen by bacteria to decompose bulk organic matter produced. Higher consumption rates of dissolved oxygen lead to oxygen deficient conditions: hypoxia ($O_2 \leq 1.4$ ml L^{-1}; 62.5 μM) or suboxia ($O_2 \leq 0.1$ ml L^{-1}; 4.5 μM) (Levin et al. 2009) in the water column. Additionally, the prevalence of freshwater layer at the surface waters as a result of intense rainfall results in strong surface stratification and acts as a barrier layer for atmospheric oxygen diffusion or supply to coastal waters contributes to oxygen depletion. Therefore, restricted replenishment from atmosphere makes the low oxygen organic-rich waters turn hypoxic. Such seasonal oxygen deficiency following the southwest monsoon upwelling, have been found along the west coast of India. The area covering oxygen levels < 0.5 ml^{-1} (or < 25 μM) is estimated to be ~1.8×10^5 km^2 where the upwelling intensity decreases from south to north (Fig 3) (Naqvi et al. 2000). High biological production during and following southwest monsoon could be due to the influx of atmospheric and river/groundwater discharges of nitrogen released by human activities, primarily agriculture.

With the collapse of this circulation, the water column becomes oxygenated in November. During the northeast monsoon (November to March), the WICC carries warmer, fresher waters of equatorial origin toward the north. The low concentrations of nutrients coupled with downwelling associated with this flow result in low productivity and relatively deep mixed layers so that the waters are well oxygenated.
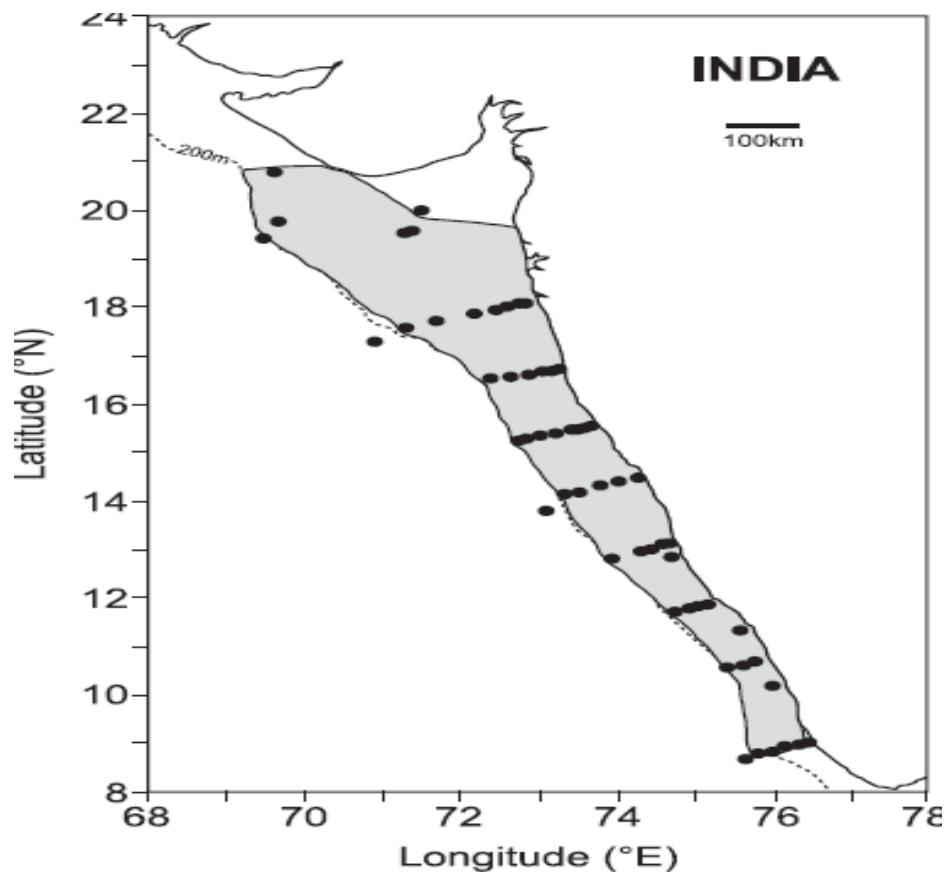
Fig 3: Shaded regions experience oxygen deficiency during the southwest monsoon

(Adapted from Naqvi et al. 2000)

2.5.3. West coast of India as a source of nitrous oxide (N_2O)

The upwelling-dominated, west coast of India is a ''hot spot'' for N_2O emissions that might make a substantial contribution to the atmospheric budget of N_2O. N_2O levels of ~765 nM (Naqvi et al. 2001; 2006) have been observed in the west coast of India. Time series observations in the last few years off Goa have clearly documented the annual occurrence of hypoxia in September-October. Thus, coastal waters are considered to be a significant source of green house gases, contributing to more than 60% of the global oceanic flux (Bange et al. 1996; Naqvi et al. 2000; 2006; 2009).

As reviewed by Naqvi et al. (2010) the west coast of India during the northeast monsoon and spring intermonsoon periods is well oxygenated, and N_2O concentration is found to be < 10 nM. As the southwest monsoon upwelling commences in June, the apparent utilization of oxygen in response to upwelling leads to an increase of NO_3^- in the subsurface layers. The concentration of N_2O begins to amass and persists as the water column turns suboxic (< 0.5 ml L^{-1}). With the rapid drop of nitrate, N_2O steeply increases thus denoting the N_2O production through denitrification.

Although bacteria are abundant and ubiquitous in marine ecosystems, relatively little is known about their diversity and composition, affected by various environmental parameters. The interaction between bacterial assemblages with their living environments shapes the bacterial community structure and affects the function of various bacterial groups. Therefore it is vital to understand the microbial ecology and its role in this eco-system.

PHYLOGENETIC ANALYSES OF CULTURABLE BACTERIA ALONG THE WEST COAST OF INDIA

3.1 <u>Introduction</u>

Coastal ecosystems are biogeochemically important regions, where organic matter undergoes intense chemical transformations. In these and all other marine ecotypes, heterotrophic bacteria are important components playing many vital roles in the cycling of most or all biologically essential elements (Cole et al. 1988). Their role is vital in the structuring of marine trophic web networks and, remineralization of organic matter (Azam et al. 1983; Azam and Long, 2001; Ducklow et al. 2002). Characterized by strong seasonal oscillations in physical forcing and biological production, the Arabian Sea is a recognized productive tropical oceanic region. Intense upwelling during south-west monsoon months of June-October results in intense biological productivity (Prasannakumar et al. 1995; Haugen et al. 2002; Prakash et al. 2007). Higher biological production during and following southwest monsoon could also be supported by the influx of atmospheric (Singh et al. 2015) and river/groundwater discharges (Shetye, 2007) of nitrogen and other nutrients brought in due to a variety of human activities.

The great demand for oxygen by bacteria to decompose the organic matter produced as a result of upwelling lead to oxygen deficient conditions with dissolved oxygen concentrations <1.42 ml L^{-1} termed as hypoxia in the water column (Wright et al. 2012). Depletion of oxygen necessitates bacteria to utilize nitrate via the denitrification process. In the denitrifying zone off the southwest coast of India, high concentrations of nitrite (>15 μM) and nitrous oxide (>500 nM) have been reported (Naqvi et al. 2000). It is this paradox that results in massive loss of fixed nitrogen effluxing nitrous oxide to the atmosphere (Bange et al. 2001; Babbin et al. 2015) which is a potent greenhouse gas that is involved in the destruction of the ozone layer. Thus, such ecosystems are hotspots for nitrogen

transformations, where due to deficiency of oxygen, nitrate serves as the main electron acceptor in organic matter remineralization.

Thus such ecotypes ought to harbor unique, diverse microbial communities in particular of heterotrophic bacteria whose metabolism controls key steps in marine biogeochemical cycling (Zehr and Ward, 2002). Because of the hydrographical and physicochemical changes along the south-west coast of India with respect to seasons, knowledge of bacterial community structure may offer required information as to understand how bacterial community differs with the changing environmental conditions. Analyses focusing on documenting bacterial community structure are essential to realize their possible functional role. Though cultivation-based methods alone cannot explore the entire microbial community, they offer the basis for elaborating the possible metabolic activities of extant microbial communities in biogeochemical cycling.

Therefore the goal of this chapter was to elucidate the Phylogenetic analyses of culture amenable bacterial communities. Following the isolation and purification of a large number of isolates the 16S rRNA gene sequencing approach was adopted to decipher the diversity and phylogenetic relatedness of culture amenable heterotrophic bacteria from the south-west coast of India which experiences seasonal hypoxia. This was done to gain an overall understanding of the culturable bacterial diversity during hypoxic and non-hypoxic periods.

3.2 Materials and methods

3.2.1 Study area

Differential solar heating across the Indian Ocean and Eurasian land mass leads the Arabian Sea to undergo seasonal reversal of wind called as the Southwest (SW) monsoon

from June to October and Northeast (NE) monsoon from November to March. The seasonal reversal of winds and currents in the west coast of India (Shetye et al. 1990) results in the mixed layer dynamics making it highly productive. Seasonal upwelling occurring along the West Coast of India (WCI) during the southwest monsoon season enriches the surface waters with nutrients (Banse, 1968) leading to increased biological production. Following this production, the degradation of the organic matter most often results in denitrification (Naqvi et al. 1994).

One of the important aspects of the south-west coast of India is the seasonal hypoxia/anoxia arising from enhanced oxygen demand for mineralization of organic matter following high surface biological production in response to upwelling. It is this feature that makes the western continental shelf of India a ''hot spot'' for N_2O (nitrous oxide) production (Naqvi et al. 2000). The West India Coastal Current which reverses seasonally (Shetye et al. 1990) being northward (November-May) and southward (June-October). These authors state that the biogeochemistry is thus different during these seasons. During summer monsoon, the upwelling-induced nutrient enrichment leads to high biological production along the south-west coast of India. Muraleedharan and Prasannakumar (1996) characterized the vertical thermal and density structures from the coastal waters Off Goa to Kochi. Their study elucidates that Off Goa, there is gentle upsloping of isotherms towards the coast bringing deeper colder waters to surface. Off Mangalore, the remarkable shoaling of thermocline towards the coast is related to active upwelling, and Off Kochi, there is pronounced deepening of isopycnals and isotherms in the sub-thermocline region.

3.2.2 Sample Collection

Water samples were collected during cruises *R V Sindhu Sankalp* (SSK 056 and SSK 079) and *R V Sindhu Sadhana* (SSD tr-008) under SIBER (Sustained Indian Ocean Biogeochemistry and Ecological Research) during October 2013, June-July 2014 and March 2015, representing the fall intermonsoon (FIM), summer monsoon (SuM) and spring intermonsoon (SIM) respectively. Sampling was carried out Off Goa, Off Mangalore and Off Kochi at three different depths (surface, mid-depth and bottom) as shown in Fig 1. Sampling locations are Off Goa: G9-0, 35, 66 m (15º37´N, 73º49´E); Off Mangalore: M8-0, 50 and 100 m (12º84´N, 74º30´E) and Off Kochi: K3-0, 40, 100m (9º96´N, 75º63´E). Water samples were collected using pre-cleaned Niskin samplers (washed with tap water and rinsed with distilled water after each sampling).

3.2.3 Measurements of nitrite, nitrate and dissolved oxygen concentrations

For each sample, dissolved oxygen (DO) was measured by the classical Winkler titration method modified by Carpenter (1965). The nutrients (nitrate and nitrite) were measured using a Skalar autoanalyser (Skalar Analytical) following standard methods (Grasshoff et al. 1983).

3.2.4 Enumeration of total bacterial counts

Subsamples of 50 ml volumes from each depth were preserved with buffered formaldehyde (2% final concentration). 2 ml of the sample was incubated with 4', 6-diamidino-2-phenylindole (DAPI; 20 µl of 1 mg ml^{-1} working solution) for 20 min and filtered onto black 0.22 µm pore-size polycarbonate membrane filters (Millipore) (Porter and Feig, 1980). Epifluorescence microscopic counts were made using a microscope (Olympus

BX-51). From each sample, 20 random microscopic fields were chosen to obtain a reliable mean.
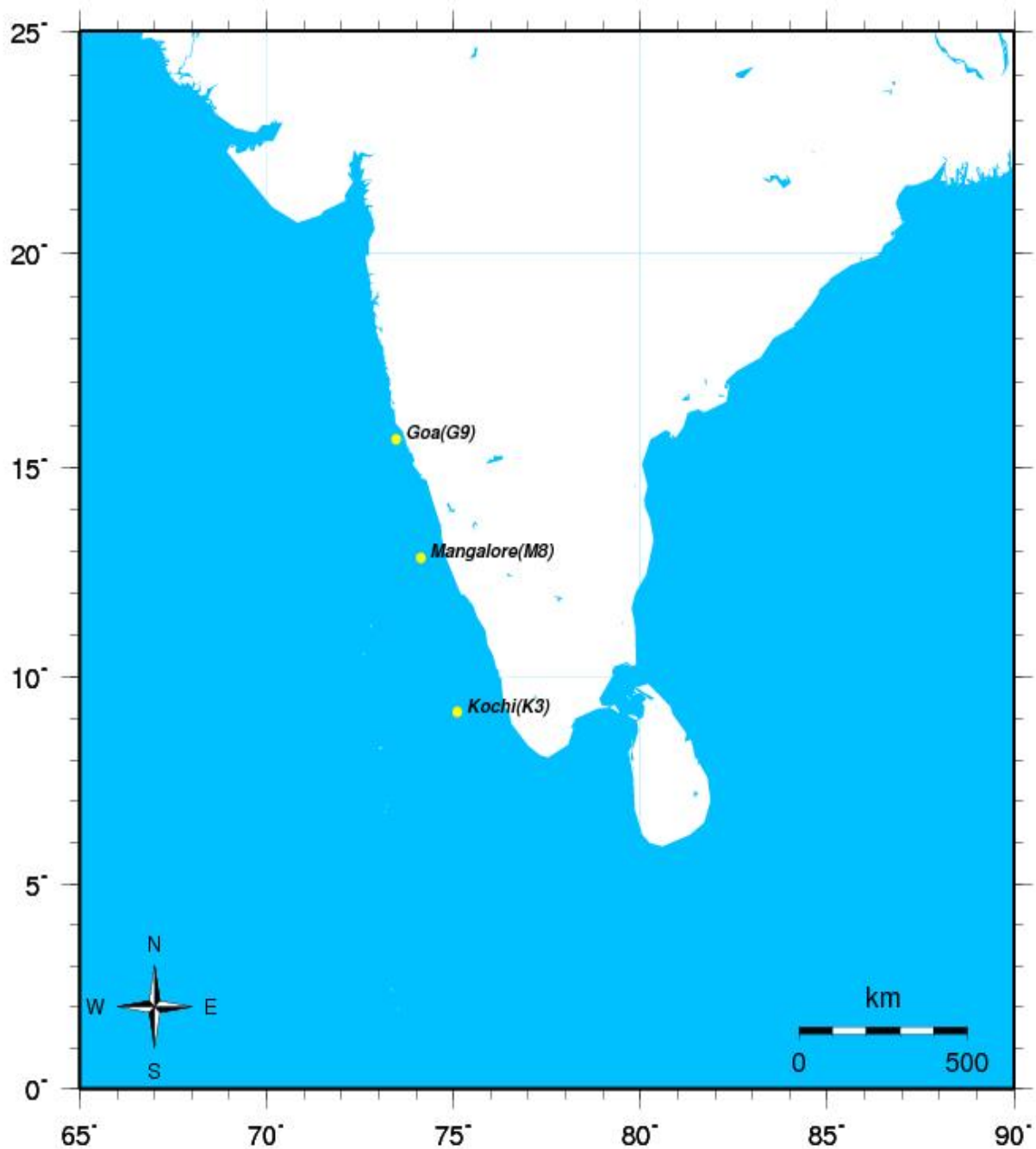
Fig. 3.1: Map of sampling locations, dots marked represents the sampling stations along the south-west coast of India

3.2.5 Isolation of culturable heterotrophic bacteria

Zobell Marine Agar (ZMA; consisting of 5 g peptic digest of animal tissue, 1 g yeast extract, 0.1 g ferric citrate, 19.45 g NaCl, 8.8 g $MgCl_2$, 3.24 g of Na_2SO_4, 1.8 g $CaCl_2$, 0.55g KCl, 0.16 g $NaHCO_3$, 0.08 g KBr, 0.034 g $SrCl_2$, 0.022 g H_3BO_3, 0.004) was used to isolate the culturable populations of aerobic, heterotrophic bacteria. Briefly, 100µl of the seawater sample was spread plated onto the ZMA plates and incubated at 28°C in an incubator on-board until visible bacterial colonies were seen. The bacterial cultures were purified by streaking well-separated colonies onto several fresh ZMA plates. Single, well-isolated colonies were picked out using a sterile microbiological loop for obtaining single colony based pure cultures. These plates were wrapped using cling-wrap, stored at 4°C, and brought back to the laboratory for their identification based on 16S rRNA sequencing technique. Morphologically distinct colonies were chosen for purification from each sample plated on to ZMA.

3.2.6 Extraction of genomic DNA from bacterial cultures

DNA was extracted from a total of 360 (Off Goa), 270 (Off Mangalore) and 270 (Off Kochi) purified cultures obtained from each season, using the modified method of DNA extraction by CTAB method as described by (William et al. 2012). The steps followed were:

1. The bacterial strains were grown overnight at 28ºC in 5 ml Zobell Marine Broth (ZMB) for 24 hours.

2. An aliquot of 1.5 ml culture was centrifuged at 10,000 rpm for 10 min in a microfuge tube. The supernatant was discarded and resuspended in 500 µl Tris-EDTA buffer.

3. To this, 40 µl of 100 mg ml⁻ lysozyme was added and incubated at 37°C for 30 min.

4. ProteinaseK (100 µl of 10 mg ml⁻), Sodium dodecyl sulfate (50 µl of 10% solution) and RNase (10 µl of 20 mg ml⁻) were added, and the tubes were incubated for 2 hours at 55°C.

5. Following this incubation, 100 µl each of 5M NaCl and CTAB/NaCl was added, mixed well and incubated at 65°C for 10 min.

6. An approximately equal volume (0.7 to 0.8 ml) of chloroform/isoamyl alcohol (24:1) was added to the above, mixed thoroughly, and centrifuged for 10 min. A white interface was visible after centrifugation.

7. The aqueous phase was collected in a fresh microfuge tube, leaving the interface behind and an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added and centrifuged for 10 min.

8. The aqueous phase was transferred in a fresh microfuge tube, 0.6 vol isopropanol was added and incubated overnight at -20°C.

9. The precipitated DNA was washed with 70% ethanol, spun at max speed for 5 min at 4°C.

10. The supernatant discarded and the pellet was allowed to dry at room temp.

11. The pellet was dissolved in 50 µl TE buffer.

12. The integrity of the total DNA was checked by agarose (0.8 %) gel electrophoresis.

3.2.7 PCR amplification of 16S rRNA gene from DNA extracts

Bacterial 16S rRNA gene from the extracted DNA was amplified by following standard polymerase chain reaction (PCR) method using universal primer set, 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492R (5´-GGTTACCTTGTTACGACTT-3´) of Weisburg et al. (1991). The PCR mixture (50 μl) contained 1 μl of extracted DNA (5-50 ng μl^{-1}), forward and reverse primer (1 μl each) at a concentration of 0.5 μM, 25 μl of Ready Mix Taq PCR mix (Sigma-Aldrich, USA) [1.5 U Taq DNA polymerase; 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl$_2$, 0.001% gelatin, 0.2 mM deoxynucleoside triphosphate (dNTP), stabilizers], and 22 μl of milliQ water. The amplification steps were as follows: initial denaturation for 10 min at 95 °C, followed by 35 cycles each for 1 min at 94 °C, at 55 °C and at 72 °C and, a final extension step for 10 min at 72 °C via Thermocycler (Applied Biosystems, USA). The PCR products thus obtained were checked by agarose gel (1.5%) electrophoresis.

3.2.8 16S rRNA gene sequencing, phylogenetic and statistical analysis

The purified PCR products of bacterial cultures containing the inserts of approximately 1500 base pairs were then sequenced by using an ABI 3130XL genetic analyzer (Applied Bio-systems). Databases (GenBank) were searched for sequences similarity analysis of the sequences obtained and compared with the NCBI database through BLAST searches (http://blast.ncbi.nlm.nih.gov). In this comparison, sequences of type strains most closely related to the sequences of the isolates were searched and checked for the chimera using DECIPHER's (http://decipher.cee.wisc.edu/) before submission. Sequences were grouped as operational taxonomic units (OTUs) by 97% or greater sequence similarity, in the MOTHUR program (Schloss et al. 2009). Representative sequences from each OTU were

aligned using the software MUSCLE (Edgar, 2004), and phylogenetic trees were constructed by the software MEGA6 using the neighbor-joining algorithm (Tamura et al. 2013). The topology of the phylogenetic tree was assessed by bootstrap analysis with 1,000 replications. Diversity indices (Coverage, Chao, Shannon, and Simpson) were calculated using MOTHUR at the cutoff level of 3% using Mothur's summary.single routine.

3.2.9 Nucleotide sequence accession numbers

Sequences obtained were submitted to GenBank and accession numbers assigned are KY742460 to KY742549, KX284748 to KX284837, KM041176 to KM041238, KT247563 to KT247589, KU878756 to KU878845, KT361410 to KT361456, KT361457 to KT361499, KY742370 to KY742459, KM041114 to KM041175, KT247505 to KT247562, KT936717 to KT936836, and KT907063 to KT907182.

3.3 <u>Results</u>

3.3.1 Environmental parameters during different seasons and locations

The environmental parameters varied widely between locations, seasons, and depths. The temperature at locations Off Goa, Off Mangalore and Off Kochi was constant (28 ℃) throughout the water column during SIM. Briefly, seasonal differences in temperature ranges were minimal with the temperature ranging from 29-30 ℃ at the surface, 21-28 ℃ in the mid-depth and 17-22 ℃ in the bottom in SuM. FIM observed 29 ℃ in the surface, 22 ℃ in the mid-depth and 19 ℃ in the close to bottom water layers, While a constant temperature of 28 ℃ throughout the water column during SIM. Dissolved oxygen (DO) varied between 194.33-252.38 μM in the surface, 27.76-150.42 μM in the mid-depth and < 70.31 μM to

undetectable levels in the samples close to the bottom in SuM. During FIM the DO ranged from 198-203.46 μM in the surface, < 50.84 μM in the mid-depth and < 27 μM to undetectable levels in the samples close to the bottom. During SIM, the entire water column was well oxygenated throughout the water column. In SuM, the nitrate concentrations ranged from an undetectable level in the surface, 2-23 μM in mid-depth to 17-26 μM in the near bottom samples. The nitrate concentration was significantly high during FIM ranging from 20-30 μM in the bottom waters, 9-30 μM in the mid-depth and negligible in the surface. Its concentrations were undetectable during SIM. Similarly, nitrite was higher in the bottom waters ranging from < 2-4 μM during FIM and undetectable levels in the surface and mid-depths. However, the nitrite values were unnoticeable during SuM and SIM. No significant difference was seen between the seasons for the TBC counts, but vertically the count was higher at the surface and low at the close to bottom depth (Table 3.1).

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Table 3.1: Season wise variations in different environmental parameters at sampling locations along the west coast of India

	Depths	SuM			FIM			SIM		
		Goa	Mangalore	Kochi	Goa	Mangalore	Kochi	Goa	Mangalore	Kochi
Temperature(°C)	Surface	30.18	29.46	26.99	29.10	29.00	29.12	29.13	28.6	28.6
	Mid	28.64	26.25	21.28	21.45	21.09	22.92	28.51	28.1	28.7
	Bottom	22.06	20.54	17.45	19.69	19.54	21.27	28.08	28.4	26.9
DO(μM)	Surface	194.33	199.03	252.38	203.46	206.10	198.87	198.06	186.59	187.48
	Mid	150.42	105.63	27.76	28.65	19.91	50.84	161.77	168.29	145.08
	Bottom	70.31	24.81	9.24	6.16	8.71	26.47	152.71	127.76	122.31
Nitrate (μM)	Surface	0.08	1.50	0.09	0.29	0.00	0.16	0.28	0.20	0.19
	Mid	0.15	2.10	23.00	29.35	30.88	9.49	0.34	0.14	0.32
	Bottom	17.01	23.04	26.82	24.86	30.79	20.67	0.00	0.00	0.00
Nitrite(μM)	Surface	0.00	0.00	0.00	0.56	0.00	0.03	0.01	0.01	0.02
	Mid	0.04	0.59	0.03	0.22	0.00	0.18	0.14	0.12	0.10
	Bottom	0.11	0.04	0.05	3.70	0.01	0.19	0.00	0.00	0.00
TBCx10^9 cells L^{-1}	Surface	10.8	11.4	10.9	11.3	10.3	11.3	10.8	9.4	9.7
	Mid	8.1	9.4	7.1	6.2	7.7	6.2	6.4	7.9	7.2
	Bottom	4.2	5.6	6.9	5.9	6.3	5.9	4.9	4.6	8.9

3.3.2 16S rRNA gene-based bacterial diversity

3.3.2.1. a Off Goa

To cover as much phylogenetic diversity of culturable bacteria as possible, over 360 cultures, at least 120 of them from each season and 40 from each sampling depth were subjected to 16S rRNA gene sequencing. From these analyses, 24 different genera were identified that belonged to five bacterial domains: *Gammaproteobacteria*, *Alphaproteobacteria, Firmicutes, Bacteroidetes*, and *Actinobacteria.* Seasonal comparisons were useful to recognize the variations in culturable bacterial species composition with respect to different seasons. Species of *Vibrio*, *Alteromonas, Marinobacter,* and *Bacillus* were found during all three seasons. However, *Bacillus* spp. was the dominant group during SIM, *Alteromonas* sp. dominated during SuM and those of *Vibrio* spp. dominated during FIM. Species of *Idiomarina, Sufflavibacter, Thalassospira,* and *Zunongwangia* were the major types during SIM and FIM.

Photobacterium and *Pseudoalteromonas* spp. were found during SuM and FIM. *Marinomonas sp.* was observed only during SuM. The species found exclusively during SIM were *Gordonia, Kocuria, Microbacterium, Erythrobacter, Psychrobacter, Chromohalobacter, Exiguobacterium,* and *Staphylococcus.* While, *Pseudomonas, Kosakonia, Salinicola, Janibacter* and *Halomonas* species were exclusively found during FIM (Fig. 3.2a). Vertical differences in the bacterial community structure between surface, mid-depth and near-bottom were also observed (Fig. 3.2b).
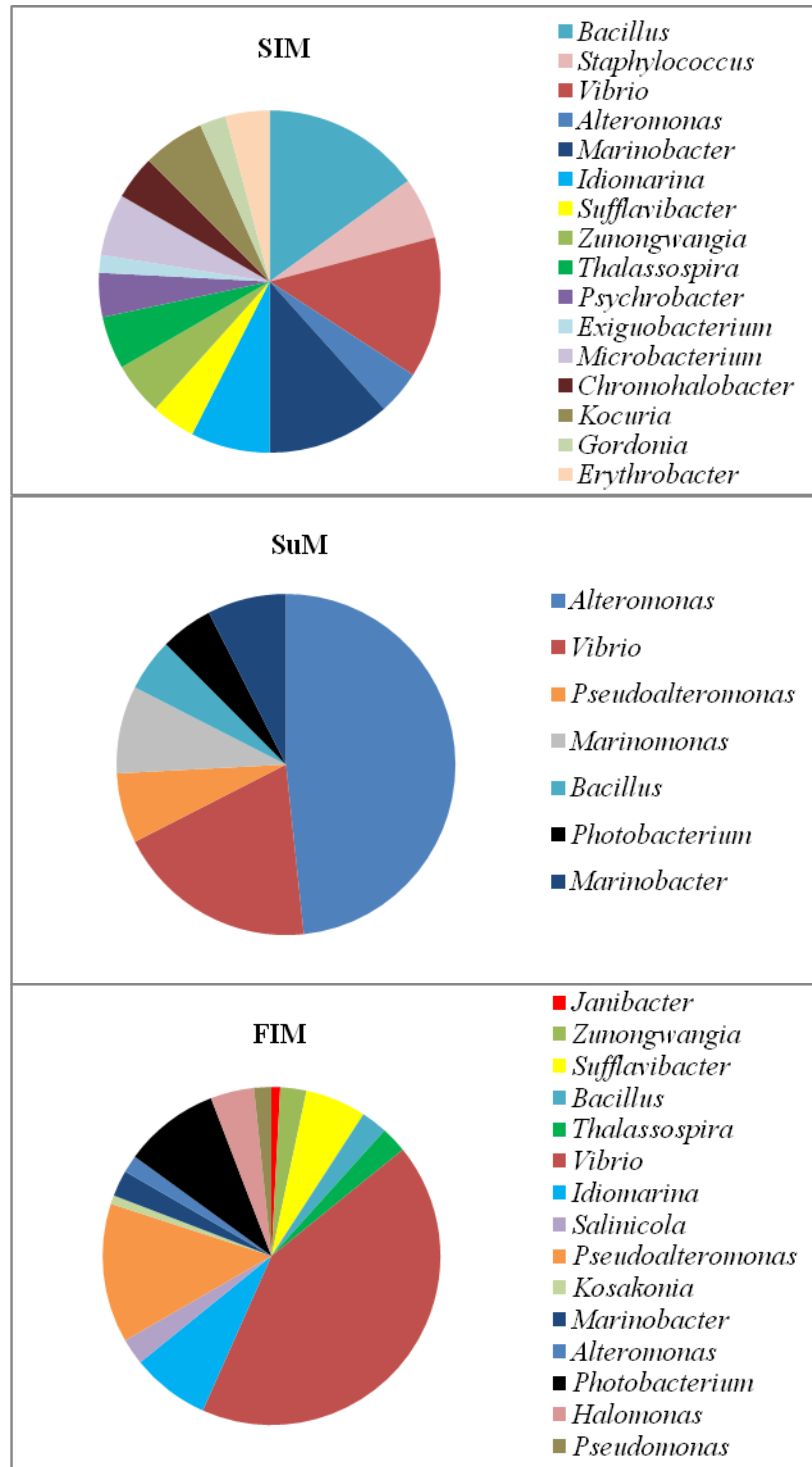
Fig.3.2a: Temporal variations in culturable bacterial community structure Off Goa

SIM

SuM

FIM
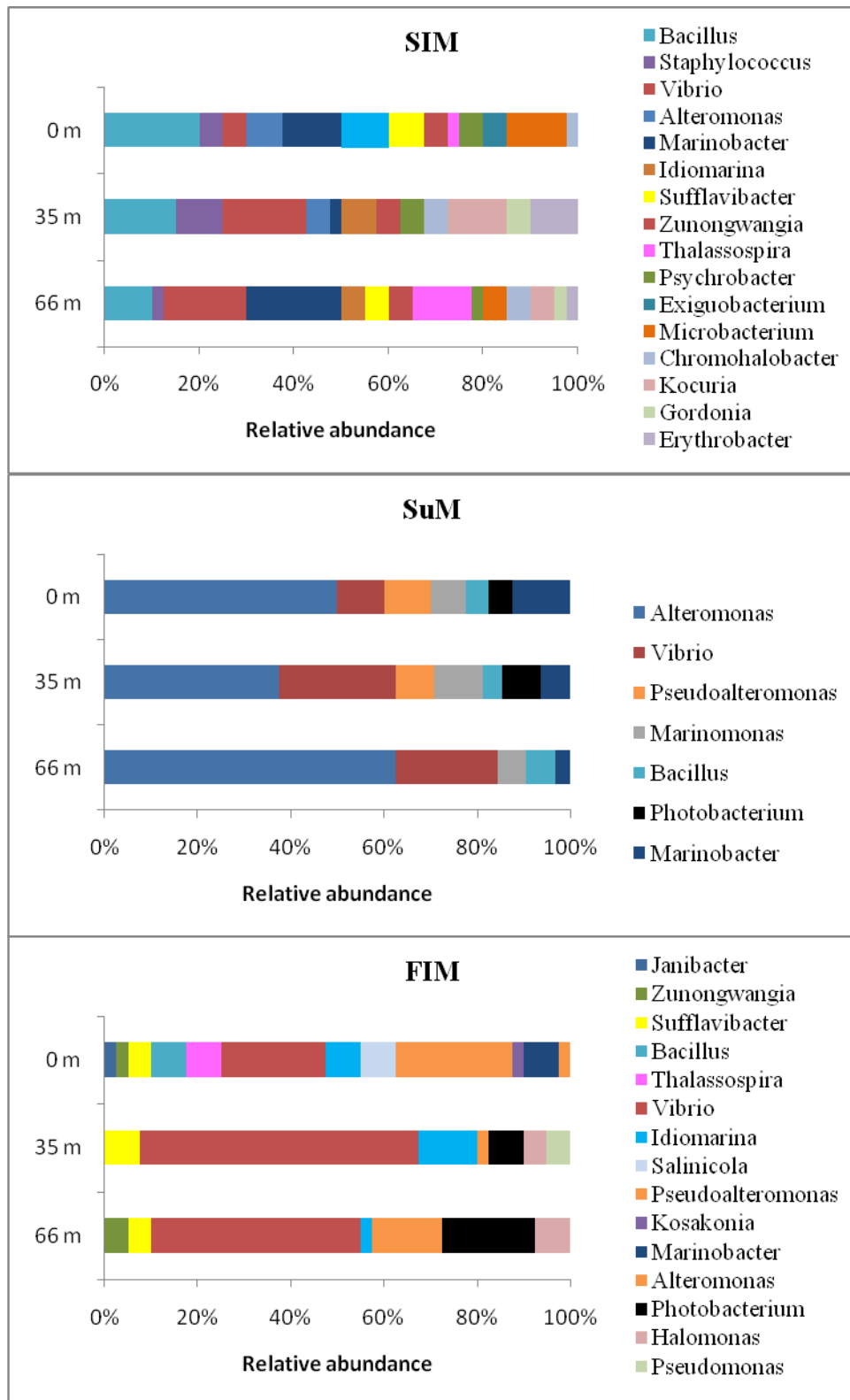
Fig.3.2b: Bacterial community structure at different sampled depths during different seasons

Off Goa

3.3.2.1. b Phylogenetic analyses

As many as 21 OTUs were formed from the 120 cultures analyzed during SIM. 10 OTUs belonged to *Gammaproteobacteria*, 4 OTUs related to *Firmicutes,* 3 OTUs related to *Actinobacteria,* 2 OTUs each representing *Alphaproteobacteria* and *Bacteroidetes*. During SuM, the 120 cultures grouped into 11 OTUs, with 9 OTUs representing *Gammaproteobacteria* and 2 belonging to *Firmicutes*. The 120 cultures analyzed during SIM grouped into 24 OTUs with 19 OTUs belonging to *Gammaproteobacteria*, 2 OTUs related to *Bacteroidetes,* and 1 OTU each representing *Alphaproteobacteria, Actinobacteria* and *Firmicutes*. A phylogenetic tree was constructed using the neighbor-joining method using MEGA 6 to show relationships between the dominant OTUs in each season and their closest neighbors (Fig 3.3 a, b and c).

3.3.2.1. c Statistical analysis

The Chao1 estimator of species richness, Shannon diversity index, Simpson diversity and Good's coverage for each sampling site were calculated across the three seasons. The Shannon diversity index was the highest during FIM and lowest during SuM with values 2.96 and 2.05 respectively. While coverage values ranged from 93% to 97% (Table 3.2). The rarefaction analysis was done for the comparing the sampling effort and phylotype obtained, indicated that more ribogroups were found during FIM and the least during SuM at equal sampling effort (Fig 3.4).

Fig 3.3a: Phylogenetic tree of culturable bacterial isolates generated from alignments of 16S rRNA gene sequences from the coastal Arabian Sea samples Off Goa during SIM and representative references retrieved from Genbank. In the bootstrap test of 1000 replicates, the percentage of the replicate trees wherein the associated taxa that clustered together is shown next to the branches. Bootstrap values > 50% is shown at each node. Scale bars represent the nucleotide substitution percentage.

Fig 3.3b: Phylogenetic tree of culturable bacterial isolates generated from alignments of 16S rRNA gene sequences from the coastal Arabian Sea samples Off Goa during SuM and representative references retrieved from Genbank. In the bootstrap test of 1000 replicates, the percentage of the replicate trees wherein the associated taxa that clustered together is shown next to the branches. Bootstrap values > 50% is shown at each node. Scale bars represent the nucleotide substitution percentage.

Vibrio rotiferianus strain (NR 118091.1) 65
Vibrio campbellii strain (NR 113782.1) 95
GFIM-OTU 1 (KM041121) 98
GFIM-OTU 2 (KT247557) 87
GFIM-OTU 3 (KT247544) 93
Vibrio chagasii strain (NR 117891.1)
GFIM-OTU 4 (KT247514) 100
Vibrio shilonii strain (NR 118242.1) 68
GFIM-OTU 5 (KM041138) 99
Vibrio brasiliensis strain (NR 117887.1) 99
GFIM-OTU 6 (KT247551) 89
Vibrio caribbeanicus strain (NR 108732.1) 85
GFIM-OTU 7 (KM041174) 100
Photobacterium leiognathi strain (NR 115206.1) 100
GFIM-OTU 8 (KM041130) 100
Kosakonia cowanii strain (NR 025566.1)
Pseudoalteromonas shioyasakiensis (NR 125458.1)
Pseudoalteromonas lipolytica strain (NR 116629.1) 100
GFIM-OTU 9 (KM041159)
GFIM-OTU 10 (KM041120) 67
GFIM-OTU 11 (KM041150) 59
Idiomarina fontislapidosi strain (NR 029115.1) 97
Idiomarina seosinensis strain (NR 025826.1)
GFIM-OTU 12 (KT247512) 100
GFIM-OTU 13 (KM041124) 100
Idiomarina sediminum strain (NR 044176.1) 99
GFIM-OTU 14 (KM041154)
Alteromonas macleodii strain (NR 114053.1) 100
GFIM-OTU 15 (KM041157) 100
Pseudomonas stutzeri strain (NR 041715.1)
GFIM-OTU 16 (KM041132) 77
Marinobacter salsuginis strain (NR 044044.1) 100
GFIM-OTU 17 (KM041151) 100
Salinicola salarius strain (NR 042490.1)
GFIM-OTU 18 (KM041155) 76
Halomonas xianhensis strain (NR 116016.1) 100
GFIM-OTU 19 (KM041162) 100
Halomonas axialensis strain (NR 027219.1) 100

Gamma proteobacteria

GFIM-OTU 20 (KM041131)
Thalassospira tepidiphila strain (NR 041492.1) 100

Alpha proteobacteria

GFIM-OTU 21 (KT247524) 100
Bacillus firmus strain (NR 112635.1)

Firmicutes

GFIM-OTU 22 (KM041163) 100
Janibacter melonis strain (NR 025805.1) 100

Actinobacteria

GFIM-OTU 23 (KM041128)
Zunongwangia profunda strain (NR 043986.1) 100
GFIM-OTU 24 (KM041135) 100
Sufflavibacter maritimus strain (NR 115876.1) 59

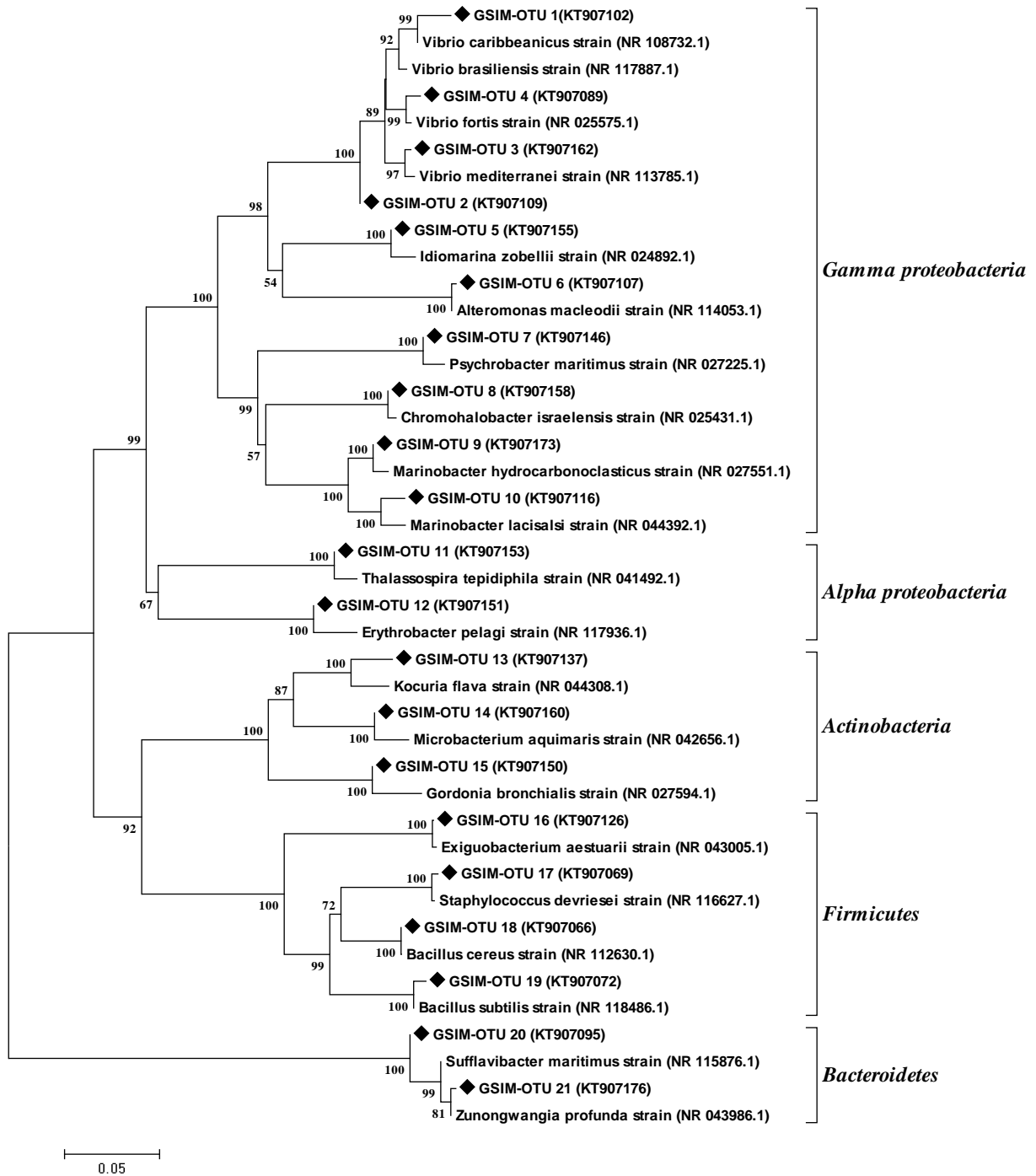*Bacteroidetes*

52 88 94 100 95 98 100 100

0.05

Fig 3.3c: Phylogenetic tree of culturable bacterial isolates generated from alignments of 16S rRNA gene sequences from the coastal Arabian Sea samples Off Goa during FIM and representative references retrieved from Genbank. In the bootstrap test of 1000 replicates, the percentage of the replicate trees wherein the associated taxa that clustered together is shown

next to the branches. Bootstrap values > 50% is shown at each node. Scale bars represent the nucleotide substitution percentage.
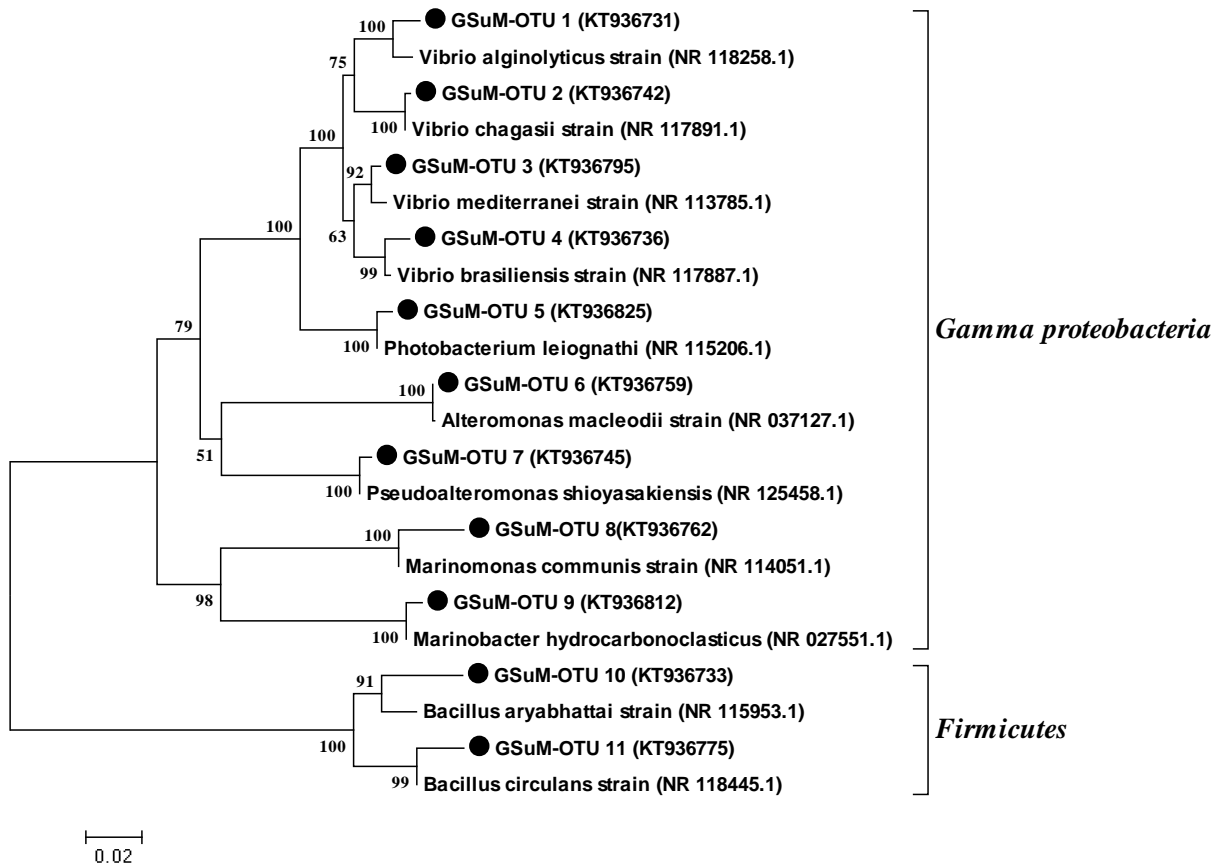
Table 3.2: Distribution of ribogroups, diversity indices and coverage of OTUs during different seasons from coastal waters of the eastern Arabian Sea Off Goa

Diagnostic	SIM	SuM	FIM
No. of cultures	120	120	120
No. of OTUs	20	11	24
Shannon's index	2.90	2.05	2.96
Simpson's index	0.05	0.21	0.06
Chao1	24.5	16	32.5
Good's coverage (%)	97	97	93

Fig 3.4: Rarefaction curves of operational taxonomic units (OTUs) of culturable bacteria obtained during different seasons.

3.3.2.2. a Off Mangalore

From the 270 cultures (at least 90 of them from each season) subjected to 16S rRNA gene sequencing, 17 different genera were identified that belonged to five bacterial domains: *Gammaproteobacteria*, *Alphaproteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*. Unlike Off Goa, *Alteromonas* sp. and *Vibrio* spp. were do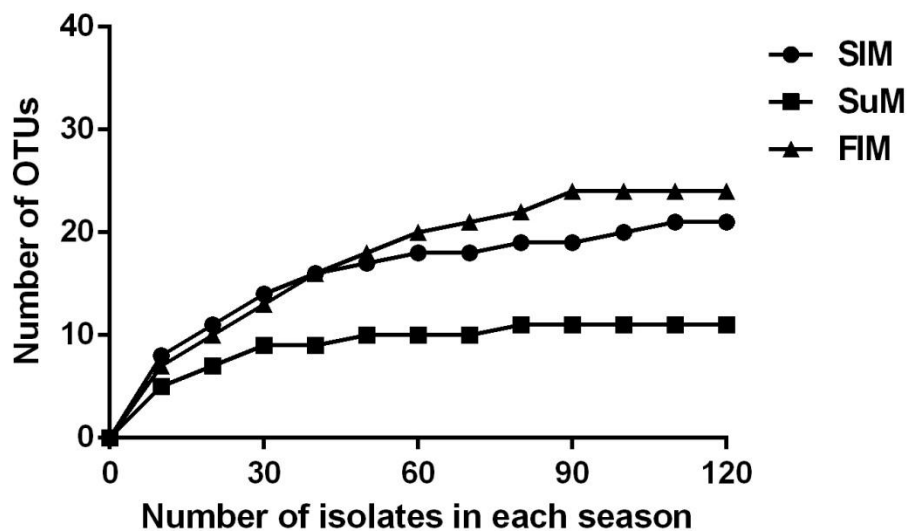minant during all three seasons. However, *Photobacterium* sp. was observed during SIM and SuM, while *Marinobacter* sp. was observed only during SuM and FIM. Species of *Sufflavibacter*, *Zunongwangia*, *Staphylococcus,* and *Bacillus* were found during SIM and FIM. Whereas, the minor groups of bacteria such as species of *Micrococcus, Grimontia, Shewanella, Macrococcus,* and *Acinetobacter* were found exclusively during SIM. *Thalassospira, Idiomarina* and *Pseudoalteromonas* sp. was observed only during FIM (Fig. 3.5a). Depth pattern of the bacterial community showed invariability between the depths. During SIM, more phylotypes were seen at the surface, while in FIM more phylotypes were seen at the mid-depth, but SuM showed a consistent pattern throughout the water column (Fig. 3.5b).

3.3.2.2. b Phylogenetic analyses

As many as 19 OTUs were formed from the 90 cultures analyzed during SIM. 10 OTUs belonged to class *Gammaproteobacteria*, 7 OTUs related to *Firmicutes,* 1 OTU each related to *Actinobacteria,* and *Bacteroidetes*. During SuM, the 90 cultures grouped into 9 OTUs representing class *Gammaproteobacteria*. The 90 cultures analyzed during FIM grouped into 14 OTUs with 10 OTUs belonging to class *Gammaproteobacteria*, 2 OTUs related to *Firmicutes* and 1 OTU each representing *Alphaproteobacteria* and *Bacteroidetes*. A phylogenetic tree was constructed using the neighbor-joining method using MEGA 6 to show

relationships between the dominant OTUs in each season and their closest neighbors (Fig 3.6 a, b and c).
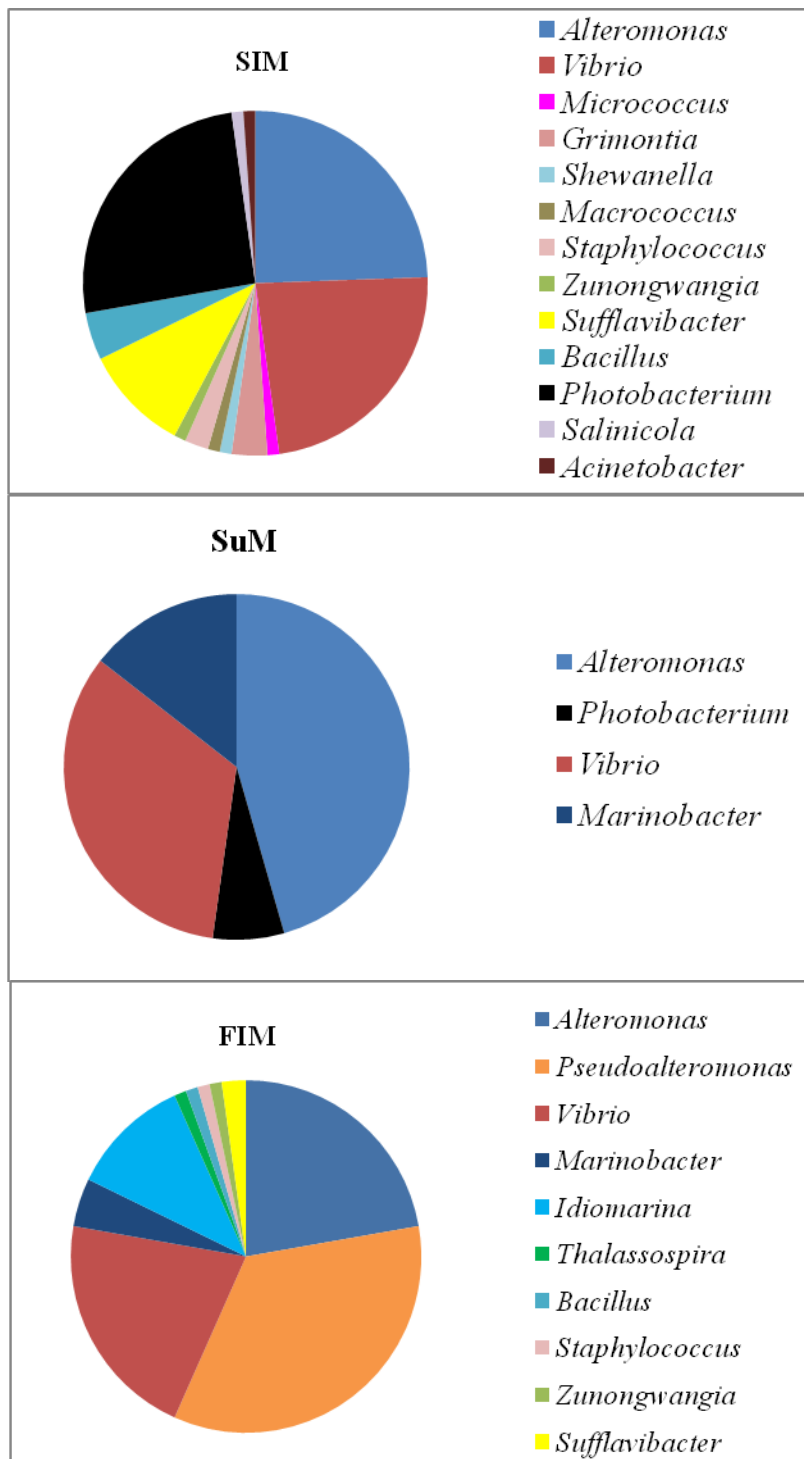
Fig.3.5a: Temporal variations in bacterial community structure Off Mangalore
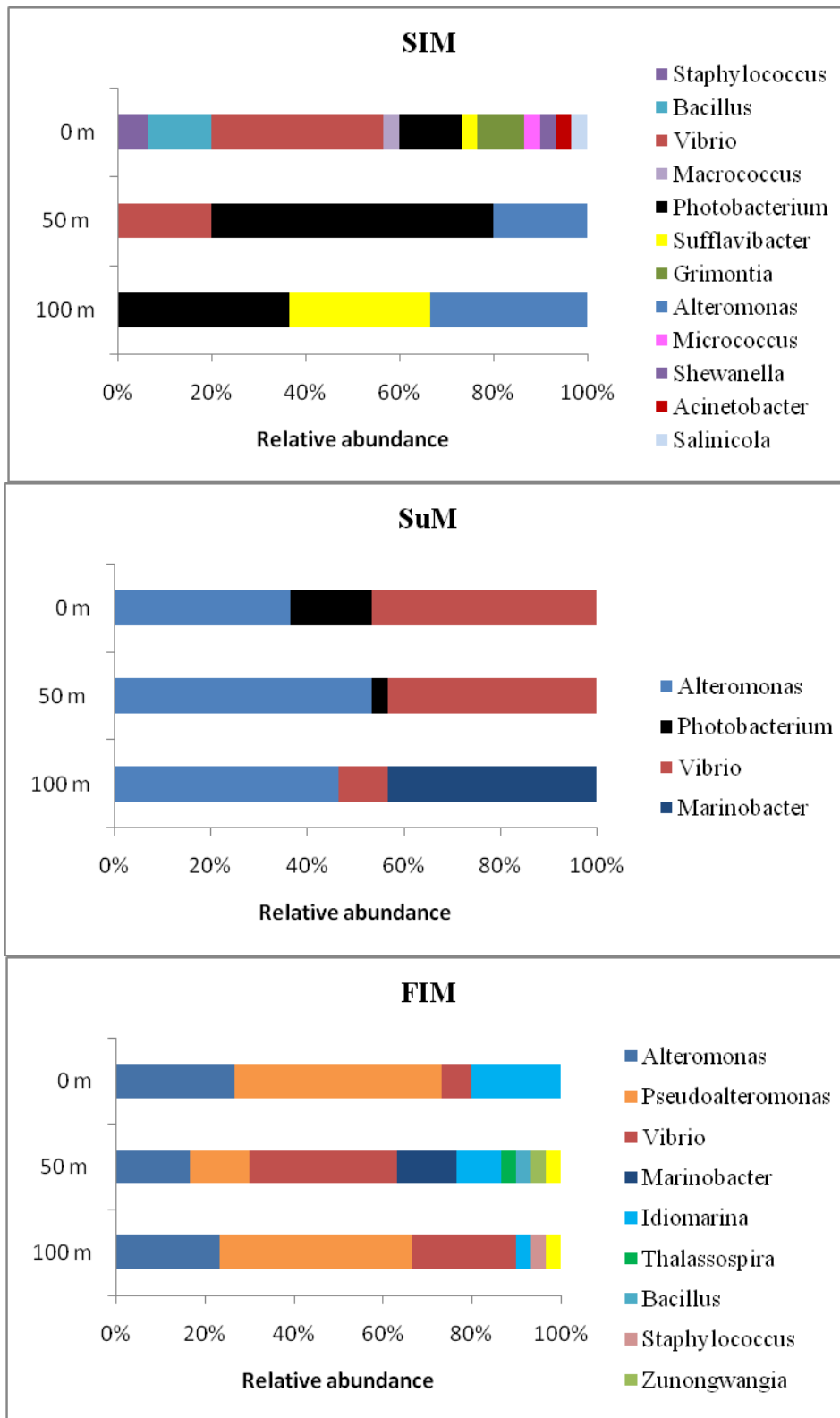
Fig.3.5b: Bacterial community structure at different depth sampled during different seasons

Off Mangalore

3.3.2.2. c Statistical analysis

Species richness, diversity indices, and the percentage of coverage in each library were determined across the three seasons. The Shannon diversity index was the highest during SIM and lowest during SuM with values 2.51 and 1.71 respectively. While coverage values ranged from 94% to 97% (Table 3.3). At equal sampling effort, the rarefaction analysis showed that more ribogroups were found during SIM and the least during SuM (Fig 3.7).
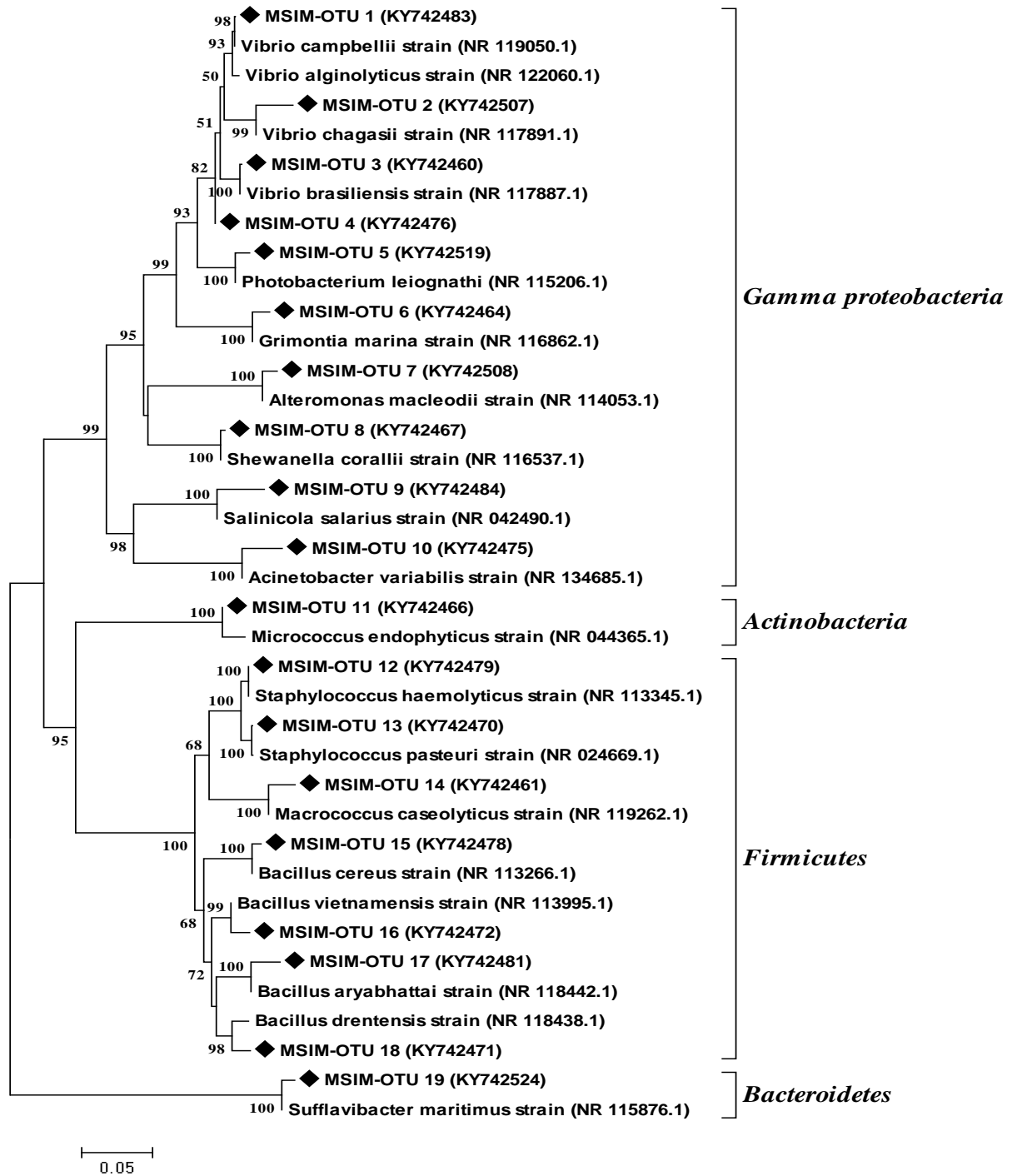
Fig 3.6a: Phylogenetic tree of culturable bacterial isolates generated from alignments of 16S rRNA gene sequences from the coastal Arabian Sea samples Off Mangalore during SIM and representative references retrieved from Genbank. In the bootstrap test of 1000 replicates, the percentage of the replicate trees wherein the associated taxa that clustered together is shown next to the branches. Bootstrap values > 50% is shown at each node. Scale bars represent the nucleotide substitution percentage.
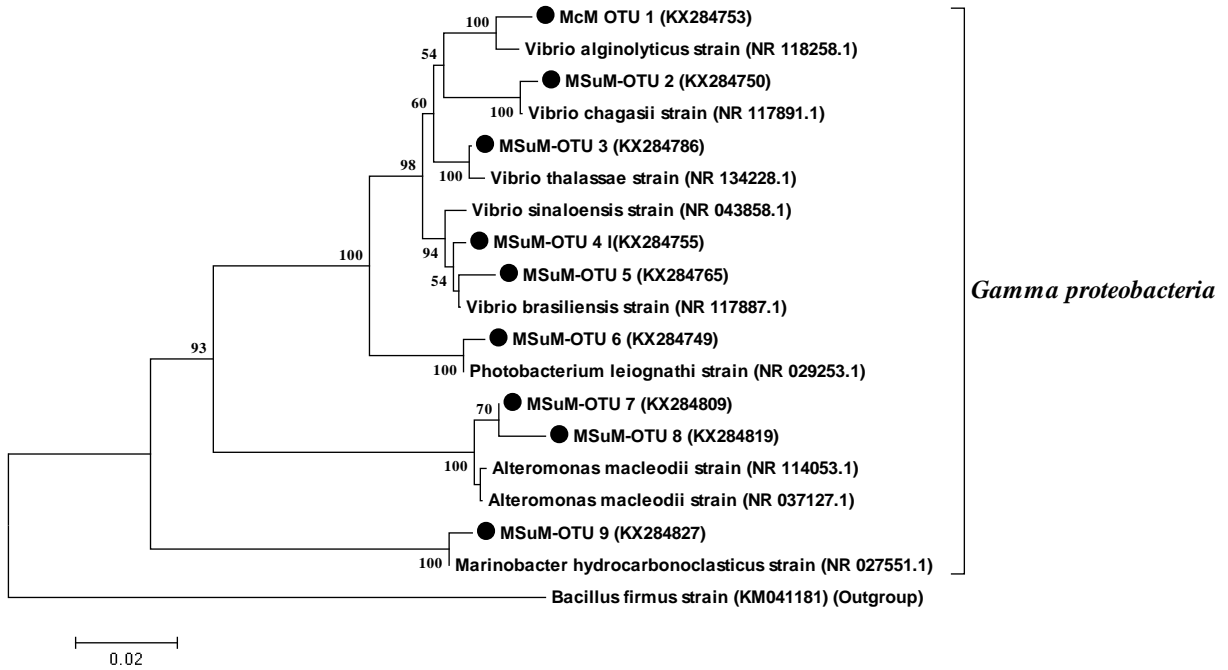
Fig 3.6b: Phylogenetic tree of culturable bacterial isolates generated from alignments of 16S rRNA gene sequences from the coastal Arabian Sea samples Off Mangalore during SuM and representative references retrieved from Genbank. In the bootstrap test of 1000 replicates, the percentage of the replicate trees wherein the associated taxa that clustered together is shown next to the branches. Bootstrap values > 50% is shown at each node. Scale bars represent the nucleotide substitution percentage.
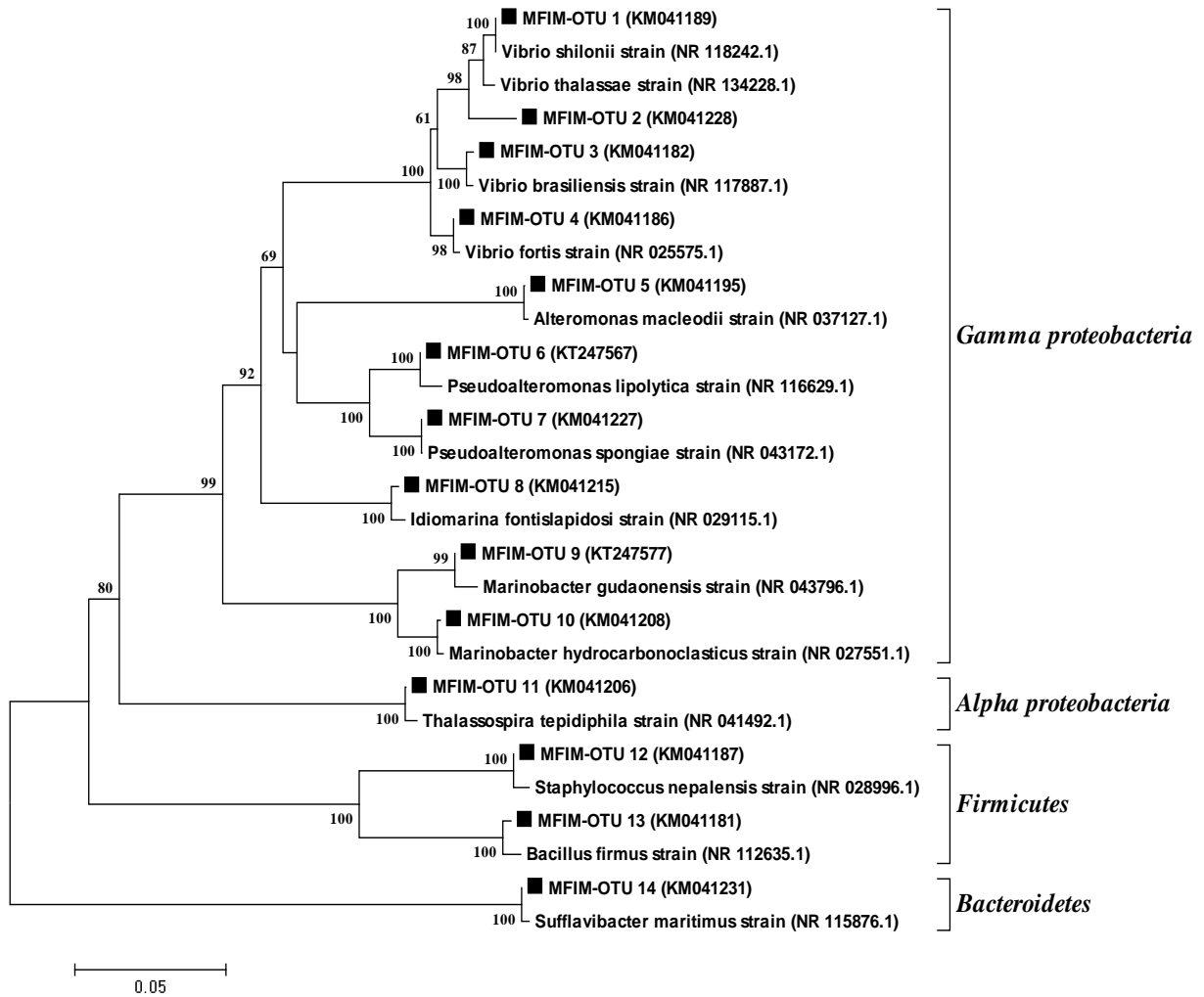
Fig 3.6c: Phylogenetic tree of culturable bacterial isolates generated from alignments of 16S rRNA gene sequences from the coastal Arabian Sea samples Off Mangalore during FIM and representative references retrieved from Genbank. In the bootstrap test of 1000 replicates, the percentage of the replicate trees wherein the associated taxa that clustered together is shown next to the branches. Bootstrap values > 50% is shown at each node. Scale bars represent the nucleotide substitution percentage.

Table 3.3: Distribution of ribogroups, diversity indices and coverage of OTUs during different seasons from coastal waters of the Arabian Sea Off Mangalore

Diagnostic	SIM	SuM	FIM
No. of cultures	90	90	90
No. of OTUs	19	9	14
Shannon's index	2.51	1.71	1.96
Simpson's index	0.10	0.24	0.18
Chao1	22.5	10	16.5
Good's coverage (%)	97	97	94
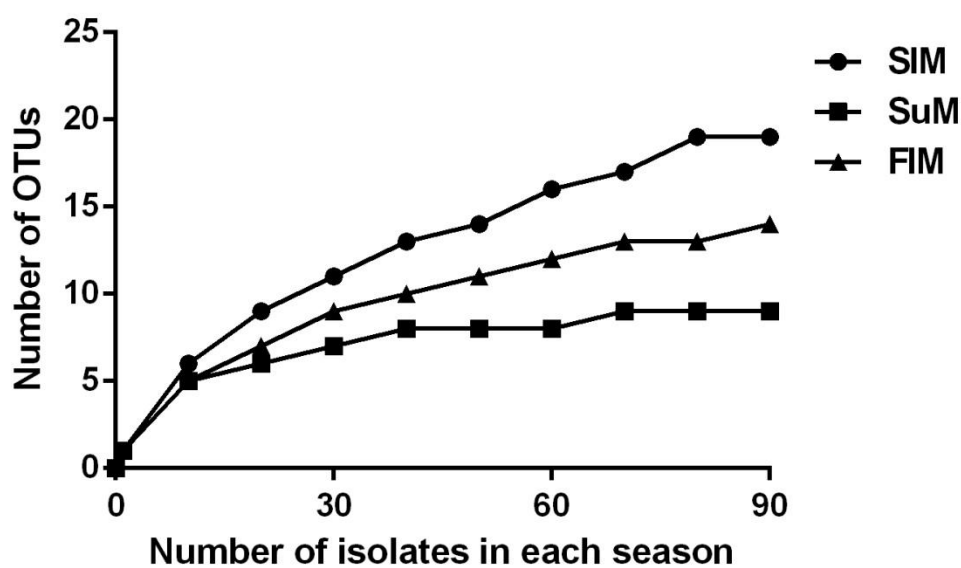
Fig 3.7: Rarefaction curves of operational taxonomic units (OTUs) of culturable bacteria during different seasons.

3.3.2.3. a Off Kochi

Based on the partial sequencing of 16S rRNA gene, 270 bacterial cultures (90 of them from each season), were identified as belonging to 13 different genera within five phylogenetic domains: *Gammaproteobacteria*, *Alphaproteobacteria*, *Firmicutes*, *Bacteroidetes,* and *Actinobacteria.*

Similar to Off Goa or Mangalore, *Alteromonas* sp. and *Vibrio* spp. were the major groups found during all the three seasons, followed by *Bacillus* spp. and *Photobacterium* sp. The species exclusively found during SIM were *Sufflavibacter, Micrococcus, Grimontia, Brevibacterium,* *Anaerobacillus,* and *Staphylococcus*, while *Marinomonas, Pseudoalteromonas,* and *Halomonas* sp. were observed only during FIM (Fig. 3.8a).

Vertical differences in the bacterial community structure between surface, mid-depth and near-bottom in the water column were also observed. The dominant bacterial groups (*Alteromonas* sp. and *Vibrio* spp.) were found throughout the water column *Bacillus* spp. were found to increase towards the bottom, on the contrary *Alteromonas* sp. were found to decrease. On the other hand, inconsistent partitioning of bacteria was observed during SIM and FIM (Fig. 3.8b).

3.3.2.3. b Phylogenetic analyses

16 different OTUs were defined among the 90 isolated strains during SIM. 7 OTUs were identified in the class *Gammaproteobacteria*, 6 OTUs were identified in *Firmicutes,* 2 OTUs related to *Actinobacteria* and 1 OTU related to *Alphaproteobacteria*. During SuM, the 90 cultures grouped into 11 OTUs, with 9 OTUs representing class *Gammaproteobacteria* and 2 OTUs representing *Firmicutes*. The 90 cultures analyzed during FIM grouped into 16 OTUs,

with 14 OTUs belonging to *Gammaproteobacteria* and 2 OTUs related to *Firmicutes*. The phylogenetic tree of dominant OTUs in each season and their closest neighbors was constructed using the neighbor-joining method using MEGA. Bootstrap tests were performed 1000 times (Fig 3.9 a, b and c).

3.3.2.3. c Statistical analysis

The community parameters: Chao1 estimator of species richness, Shannon diversity index, Simpson diversity and Good's coverage for each sampling site were calculated across the three seasons. It was seen that Shannon diversity index was lowest during SuM (1.71) and no significant difference was seen in the diversity indices during SIM and FIM. While coverage values ranged from 93% to 98% (Table 3.4). The rarefaction analysis indicated that more ribogroups were found during FIM and least during SuM at equal sampling effort (Fig 3.10).
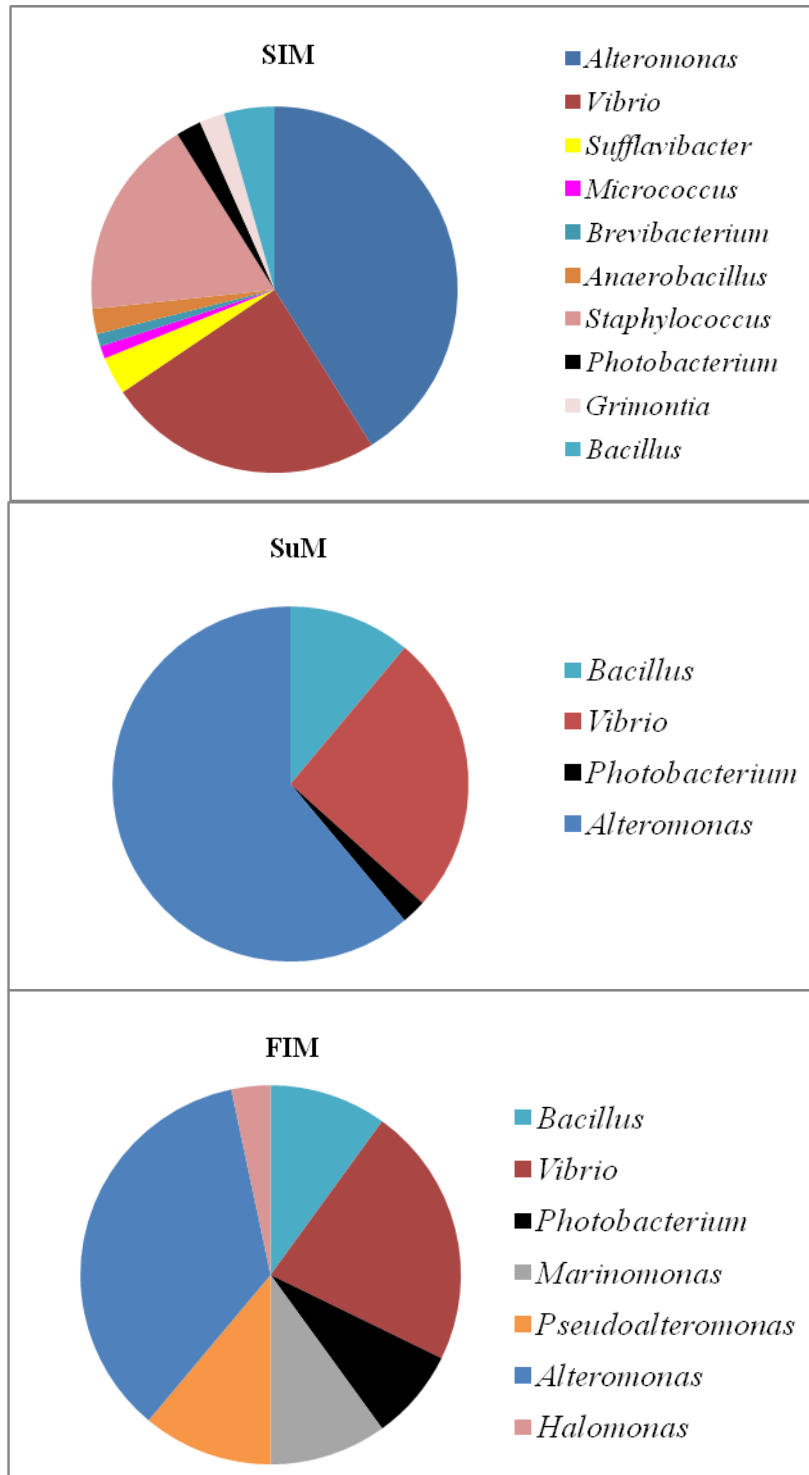
Fig.3.8a: Temporal variations in culturable bacterial community structure Off Kochi
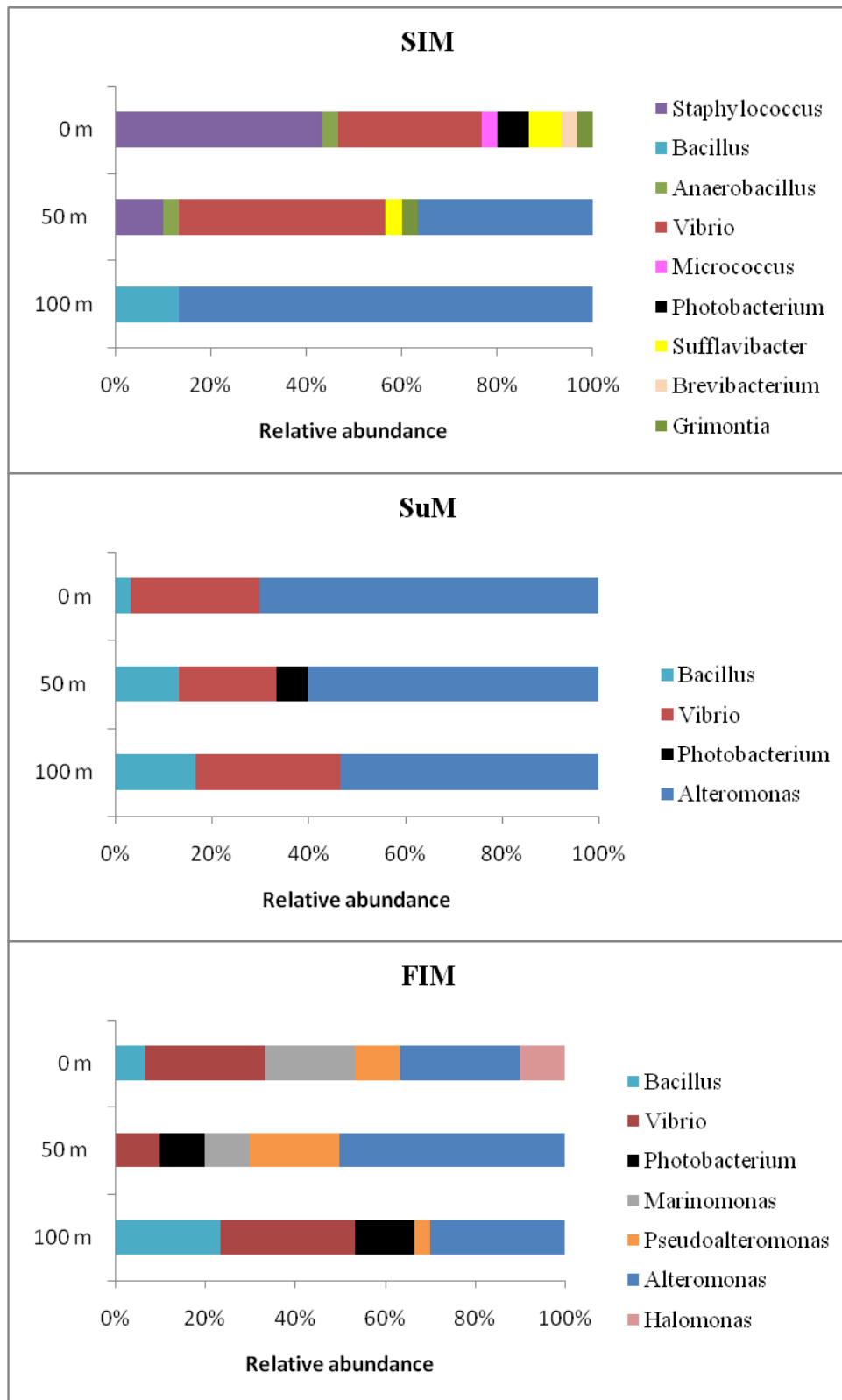
Fig.3.8 b: Bacterial community structure at different sampled depths during different seasons Off Kochi
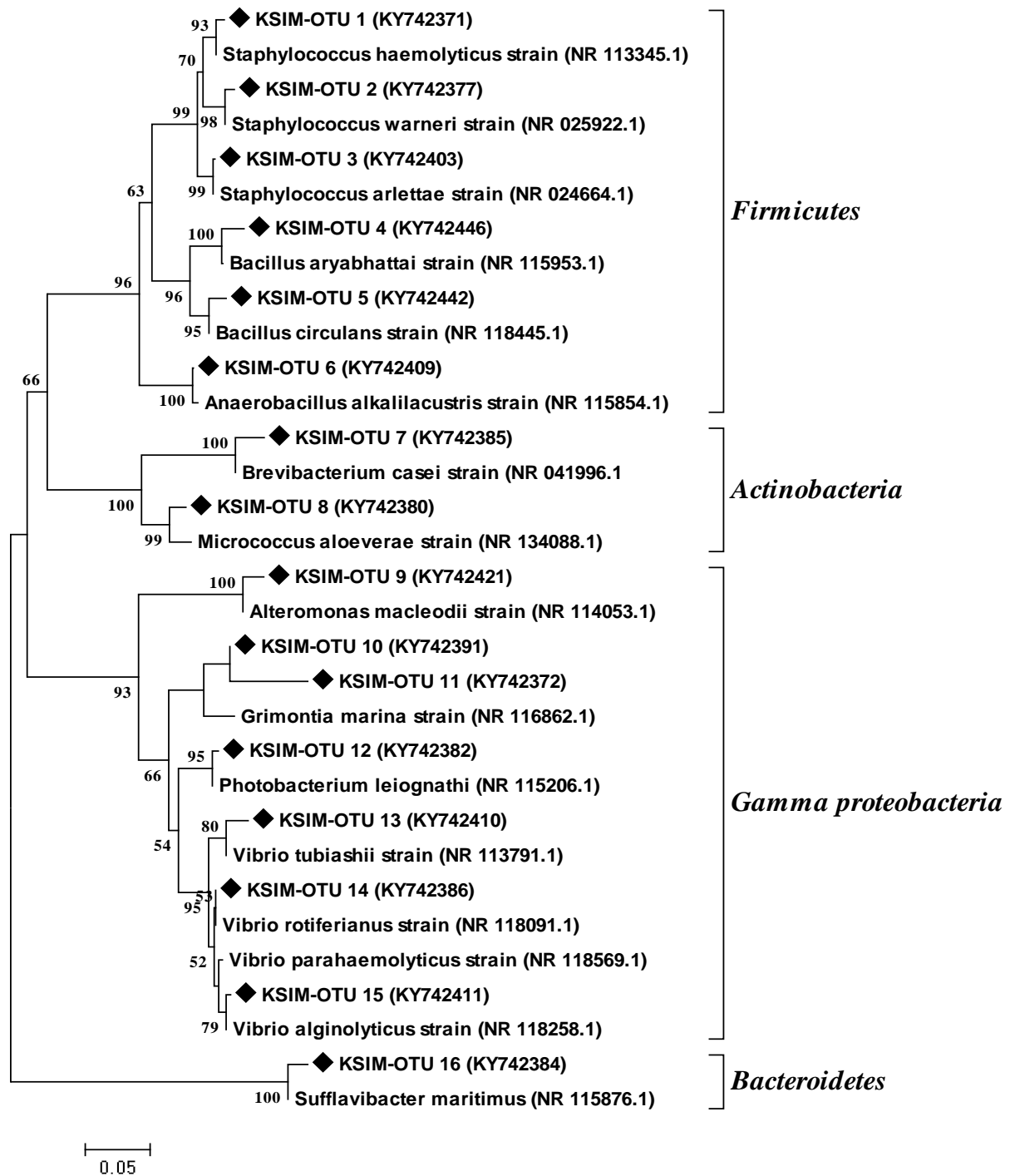
Fig 3.9a: Phylogenetic tree of culturable bacterial isolates generated from alignments of 16S rRNA gene sequences from the coastal Arabian Sea samples Off Kochi during SIM and representative references retrieved from Genbank. In the bootstrap test of 1000 replicates, the percentage of the replicate trees wherein the associated taxa that clustered together is shown next to the branches. Bootstrap values > 50% is shown at each node. Scale bars represent the nucleotide substitution percentage.
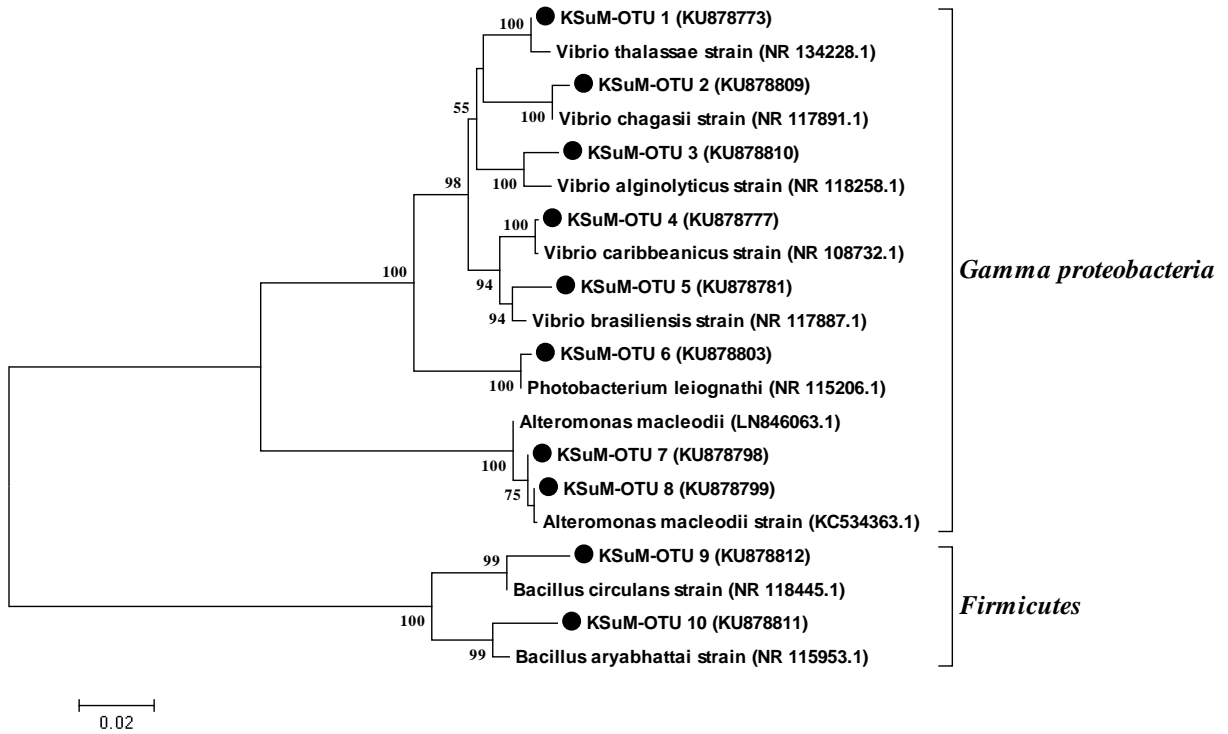
Fig 3.9b: Phylogenetic tree of culturable bacterial isolates generated from alignments of 16S rRNA gene sequences from the coastal Arabian Sea samples Off Kochi during SuM and representative references retrieved from Genbank. In the bootstrap test of 1000 replicates, the percentage of the replicate trees wherein the associated taxa that clustered together is shown next to the branches. Bootstrap values > 50% is shown at each node. Scale bars represent the nucleotide substitution percentage.
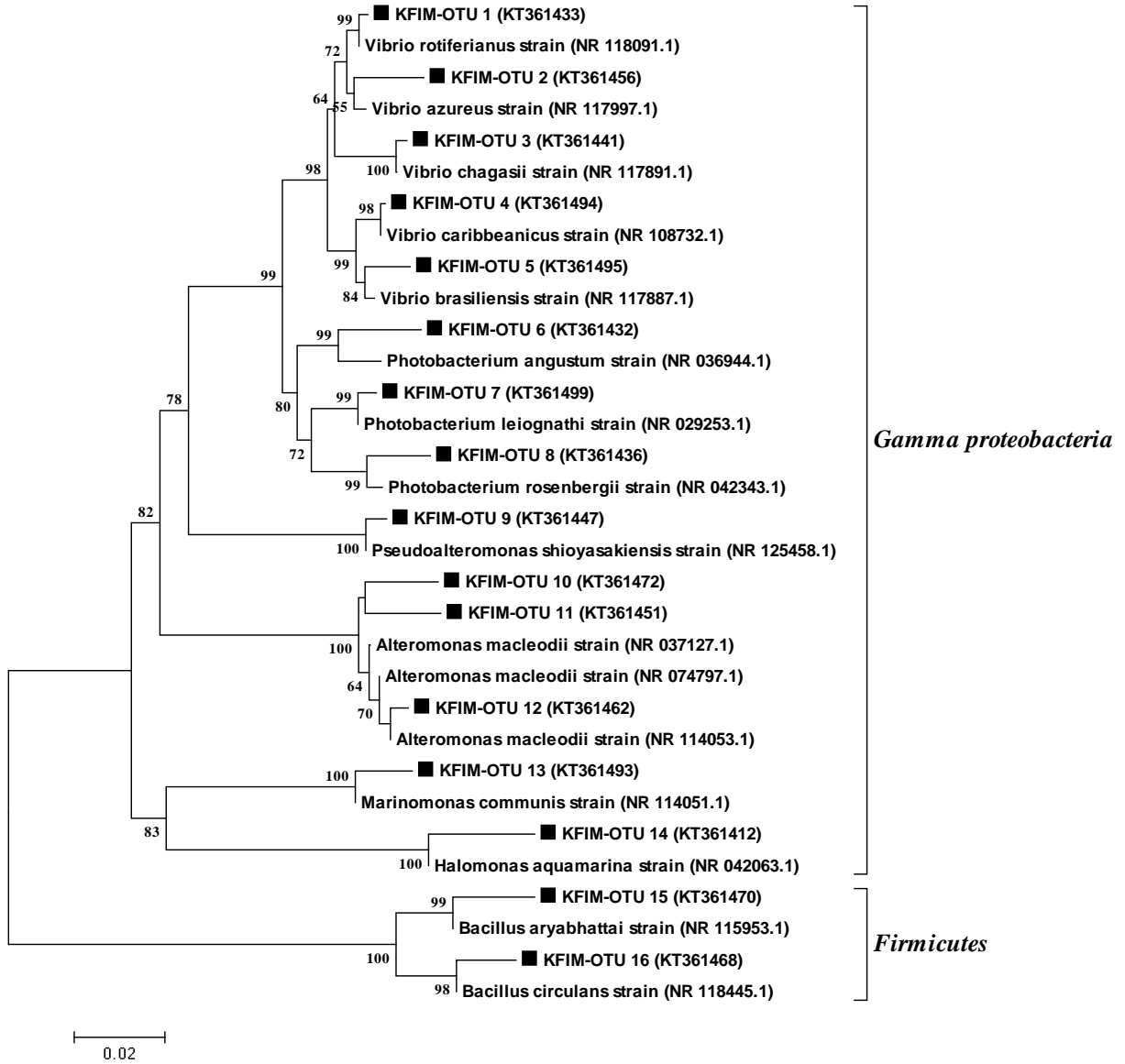
Fig 3.9c: Phylogenetic tree of culturable bacterial isolates generated from alignments of 16S rRNA gene sequences from the coastal Arabian Sea samples Off Kochi during FIM and representative references retrieved from Genbank. In the bootstrap test of 1000 replicates, the percentage of the replicate trees wherein the associated taxa that clustered together is shown next to the branches. Bootstrap values > 50% is shown at each node. Scale bars represent the nucleotide substitution percentage.

Table 3.4: Distribution of ribogroups, diversity indices and coverage of OTUs during different seasons from coastal waters of the Arabian Sea Off Kochi

Diagnostic	SIM	SuM	FIM
No. of cultures	90	90	90
No. of OTUs	16	10	16
Shannon's index	2.20	1.75	2.24
Simpson's index	0.18	0.24	0.14
Chao1	21	10	18
Good's coverage (%)	93	98	95
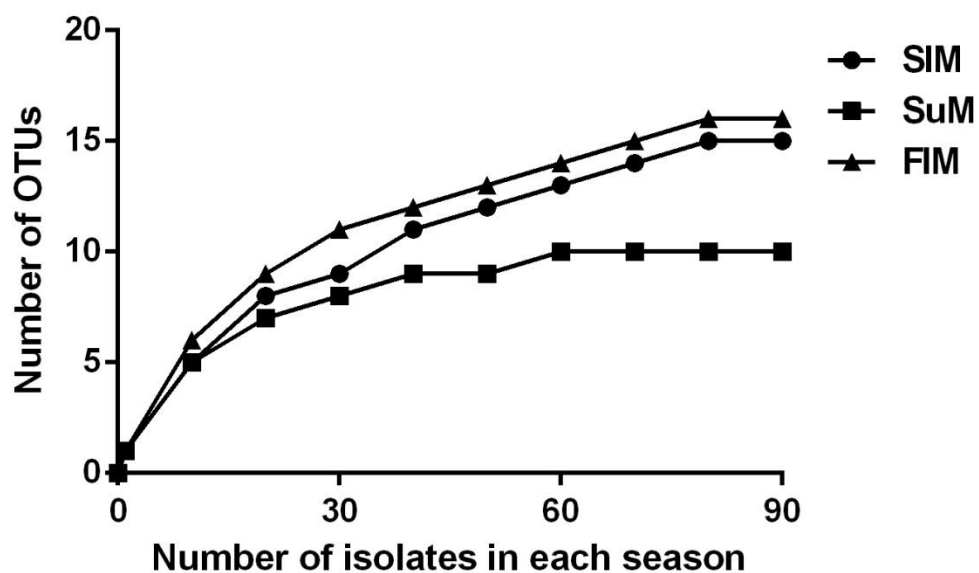
Fig 3.10: Rarefaction curves of operational taxonomic units (OTUs) of culturable bacteria obtained during different seasons.

3.4 <u>Discussion</u>

3.4.1 Physical and biogeochemical characteristics of West coast of India

In this study, it was observed that during summer monsoon (SuM: June-August) and Fall Intermonsoon (FIM: September-October) seasons, the temperature and dissolved oxygen concentrations steeply decrease in the mid-depth and bottom layers. Corresponding to this decrease, the nitrate and nitrite concentrations increase. However, constant temperature and Dissolved Oxygen (DO) was constant throughout the water column during Spring Intermonsoon (SIM: March-May); and concentration of nitrogen species was close to zero/undetectable. This is because, during SuM and FIM the mid and bottom water temperature dropped significantly due to the upwelling of cold water. However, the presence of a freshwater layer over the subsurface layer following rainfall prevented further vertical migration of this upwelled water. During the southwest monsoon upwelling pumps nitrate rich waters into the photic layers leading to intense biological productivity, the degradation of organic matter sinking from productive surface waters depletes oxygen during SuM leading to hypoxia in the subsurface water ($O_2 \leq 1.4ml^{-1}$; 62.5 μM) and the low oxygen conditions further intensifies during FIM resulting in suboxia ($O_2 \leq 0.1ml^{-1}$; 4.5 μM). This necessitates bacteria to use alternative electron acceptor thereby leading to denitrification. The upwelling collapses by late October and downwelling that occurs off the WCI during SIM, the water column is observed to be well-mixed and hence constant temperature was found throughout the water column. The DO was uniformly high at all depths during the SIM which was because of the well mixed water column. During these 7-8 months, the concentrations of nitrate are generally low (Pratihary, 2008) as a result of higher photosynthetic uptake under favorable hydrochemical conditions.

3.4.2 Comparison of bacterial communities in oxygen minimum zones

Studies have widely reported the presence of unculturable fractions from hypoxic regions and oxygen minimum zones. Divya et al. (2011) studied the culturable bacteria by 16S rRNA gene sequencing from the sediments of oxygen minimum zones in the Arabian sea and reported the presence of three phylogenetic groups: *Firmicutes* (*Bacillus* sp., *Virgibacillus* sp., *Paenibacillus* sp., *Halobacillus* sp., *Marinilactibacillus* sp.), *Gammaproteobacteria* (*Halomonas* sp. and *Alteromonas* sp., *Actinobacteria* (*Kytococcus* sp. and *Micrococcus* sp.), low G+C bacteria and unidentified bacteria.

In contrast, the culturable bacteria by 16S rRNA gene sequencing in this study reported the presence of five bacterial lineages: *Gamma proteobacteria* (Species of *Vibrio, Alteromonas, Pseudoalteromonas, Marinobacter, Idiomarina, Photobacterium, Marinomonas, Halomonas, Pseudomonas, Psychrobacter, Salinicola, Kosakonia, Chromohalobacter, Grimontia, Shewanella* and *Acinetobacter*), *Alpha-proteobacteria* (*Thalassospira* and *Erythrobacter* sp.), *Bacteroidetes* (*Sufflavibacter* and *Zunongwangia* sp.), *Actinobacteria* (Species of *Janibacter, Gordonia, Microbacterium, Kocuria, Micrococcus*) and *Firmicutes* (Species of *Bacillus, Anaerobacillus, Macrococcus* and *Staphylococcus*).

3.4.3 Bacterial community differences between off Goa, Mangalore and Kochi

The major phylotypes comprised of *Altermonas* and *Vibrio* sp. were found at all the three locations (Off Goa, Off Mangalore and Off Kochi), followed by species of *Photobacterium, Sufflavibacter, Pseudoalteromonas, Staphylococcus* and *Bacillus. Zunongwangia, Thalassospira, Salinicola, Marinobacter* and *Idiomarina* sp. were found only off Goa and off Mangalore while *Marinomonas* and *Halomonas* sp. were found only off Goa

and off Kochi. *Grimontia* and *Micrococcus* sp. were observed only off Mangalore and off Kochi.

Certain minor bacterial strains sharing no spatial commonness were as follows:

Off Goa: *Psychrobacter* sp., *Exiguobacterium* sp., *Microbacterium* sp., *Chromohalobacter* sp., *Kocuria* sp., *Gordonia* sp., *Erythrobacter* sp., *Janibacter* sp., *Kosakonia* and *Pseudomonas* sp.

Off Mangalore: *Shewanella* sp., *Macrococcus* sp. and *Acinetobacter* sp.

Off Kochi: *Brevibacterium* sp. and *Anaerobacillus* sp.

3.4.4 Roles of different bacterial domains

The chimera free 16S rRNA gene sequences of cultures in the WCI bacteria belonged to five bacterial lineages: *Gamma-* and *Alpha-proteobacteria, Bacteroidetes, Actinobacteria,* and *Firmicutes.*

Gammaproteobacteria

Cultures belonging to *Gammaproteobacteria* were predominant and most were closely related to genera such as *Vibrio, Alteromonas*, *Pseudoalteromonas*, *Marinobacter, Idiomarina, Photobacterium, Marinomonas, Halomonas, Pseudomonas, Psychrobacter*, *Salinicola, Kosakonia, Chromohalobacter, Grimontia, Shewanella* and *Acinetobacter.*

Bacteria from this phylogenetic group are among the most recognized and readily cultivable microorganisms from the marine environment (Fuhrman and Hagström, 2008), and this result is in agreement with reports from other studies on diverse marine environments experiencing oxygen deficiency as well as coastal waters (Stevens and Ullao, 2008; Wietz et

al. 2010; Divya et al. 2011; Ghiglione and Murray, 2012). Widely distributed throughout the marine environment, *Alteromonas macleodii* are copiotrophs involved in the processing of dissolved organic matter was found to be dominant in this study. As reported by López-Pérez et al. (2012) the upwelling rich waters and convective mixing favor the growth of *A. macleodii,* this was evident in this study throughout the three seasons. Also, its microaerophilic adaptation to use nitrate reductase sustains its growth when oxygen is limiting.

The next dominant bacteria in this study were *Vibrio* spp. *Vibrio* species are normal residents in coastal waters playing an important role in biodegradation, nutrient regeneration and biogeochemical cycling (Thompson et al. 2004). They are significant in remineralization of organic matter in the sea (Fukami et al. 1985) and occur mainly in warmer waters (Wright et al. 1996). Increasing seawater temperature associated with global climate change is known to promote proliferation of *Vibrio* thereby causing *Vibrio*-associated diseases (Harvell et al. 2002, Vezzulli et al. 2012, Baker-Austin et al. 2013, Tout et al. 2015). In addition to this, the intense productivity in the Arabian Sea also favors the proliferation of *Vibrio* (Hsieh et al. 2007) The broad metabolic range of *Vibrio* and its ability to use different available nutrient resources by producing variety of enzymes (Thompson and Polz, 2006) enables them to survive in any extreme conditions in response to changing environment by switching from an active stage to a dormant, viable but not culturable (VBNC) stage; yet they may still be very potent opportunists if favorable conditions recur (Colwell et al. 1985, McDougald and Kjelleberg 2006). McCarren et al. (2010) found *Idiomarina* sp. and *Alteromonas macleodii* in metabolizing semilabile high molecular weight dissolved organic matter to methanol or formaldehyde, and carbon dioxide. *Pseudoalteromonas* sp. are reported to be in association

with marine eukaryotes (Skovhus et al. 2007) and are known to produce agarolytic enzymes. *Pseudoalteromonas* and *Idiomarina* were reported from the oxygen minimum zone in the central Arabian Sea (Jain et al. 2014). Studies carried out on culturable bacteria isolated from the Arabian Sea sediments also reported the presence of *Vibrio, Alteromonas, Pseudomonas, Halomonas, Photobacterium, Psychrobacter* species (Divya et al. 2011; Mendez et al. 2013; Ramya et al. 2013). *Marinobacter hydrocarbonoclasticus* is reported to degrade the hydrocarbons and can use nitrate as the terminal electron acceptor (Gauthier et al. 1992). Further, *Pseudomonas stutzeri,* the common denitrifying bacterium as reported by Carlson and Ingraham, (1983) and Deng et al. (2014) are capable of degrading a number of organic pollutants.

Other minor groups included species of *Psychrobacter, Chromohalobacter, Shewanella, Grimontia, Halomonas, Acinetobacter, Kosakonia, Marinomonas,* and *Salinicola*. Transcriptomics of psychrophilic bacteria *Psychrobacter* revealed its adaptation to significant variations of temperature (Bergholz et al. 2009), thereby adapting to environmental warming. Global warming is reducing the degree of cold habitats thus affecting the evolution of psychrophiles. *Psychrobacter* spp. are found in aquatic environments contaminated with hydrocarbon (Harwati et al. 2007; Azevedo et al. 2013). Also, this genus is known to denitrify (Zhang et al. 2011). Studies have reported the denitrifying genes to be present in *Halomonas* sp. (González-Domenech et al. 2010). *Chromohalobacter*, *Grimontia* and *Shewanella* sp. have been found to reduce nitrate (Sanchez-Porro et al. 2007; Shnit-Orland et al. 2010; Choi et al. 2012). As reported by Su et al. (2015) *Acinetobacter* sp. capable of nitrification as well as denitrification was also observed in this study.

Alphaproteobacteria

Thalassospira and *Erythrobacter* sp. belonging to *Alphaproteobacteria* were isolated in this study. The low frequency of cultured *Alphaproteobacteria* was reported earlier (Basu et al. 2013) in Northern Arabian Sea. Kodama et al. (2008) observed that *Thalassospira* sp. could grow facultatively anaerobically using nitrate as the final electron acceptor and degraded polycyclic aromatic hydrocarbon. *Erythrobacter* is a known anoxygenic phototrophic bacterium contributing to the marine carbon cycling (Kolber et al. 2001). Beazley et al. (2012) presumed it to be contributing to aromatic hydrocarbon degradation.

Bacteroidetes

Members of the phylum *Bacteroidetes* are important heterotrophs involved in cycling organic carbon in aquatic habitats (O'Sullivan et al. 2006) and are often abundant in nutrient-rich waters. *Sufflavibacter* and *Zunongwangia* sp. belonging to *Flavobacteriaceae* were cultivable in this study. The family *Flavobacteriaceae* within this phylum has been considered to have a positive correlation with the organic matters and play an important role for the degradation of polymeric substances derived from algal biomass (Bauer et al. 2006). Qin et al. (2010) reported *Zunongwangia* sp. from sediments to degrade organic nitrogen.

Actinobacteria

Bacterial species belonging to the phylum *Actinobacteria* (*Janibacter*, *Gordonia*, *Microbacterium*, *Kocuria,* and *Micrococcus*) were also found in this study. *Actinobacteria* are the substantial part of the bacterial community in the coastal waters in the Arabian Sea (Singh et al. 2011, Basu et al. 2013). Ahmed et al. (2010) and Khessairi et al. (2014) reported

biodegradation of recalcitrant compounds and toxic polycyclic aromatic hydrocarbons by species of *Kocuria* and *Janibacter*. Qin et al. (2017) reported the degradation of polycyclic aromatic hydrocarbon by *Microbacterium* sp. under denitrifying conditions. Zhang et al. (2015) reported the presence of denitrifying genes in *Microbacterium* sp. *Kocuria* and *Micrococcus* sp. have been found to reduce nitrate (Zhou et al. 2008; Chen et al. 2009).

Firmicutes

Firmicutes reported in this study were *Bacillus, Anaerobacillus, Macrococcus* and *Staphylococcus* species respectively. *Bacillus* sp. were more abundant in the sediments of the Arabian Sea responsible for organic matter mineralization; thereby playing important role in *in situ* biogeochemical processes (Divya et al. 2011). The great metabolic and physiological diversity of *Bacillus* sp. in addition to producing endospores allows their distribution in all environment of our planet (Priest, 1993). Denitrification is reported to be a common feature among members of genus *Bacillus* (Verbaendert et al. 2011; Saïd et al. 2014). *Macrococcus caseolysticus* observed in this study is reported to possess genome for nitrate reduction (Baba et al. 2009).

Da Silva et al. (2013) noted that phylotypes of *Firmicutes* and *Actinobacteria* largely comprise the gram-positive bacteria and, they are mainly reported in marine sediments. The presence of hydrocarbons in the continental margin areas of the WCI has already been the attention of many of the geochemical investigations (Paropkari et al. 1993). Hydrocarbons in the environment bring about adaptive responses in microbial communities resulting in a net increase in the number of hydrocarbon-utilizing organisms. The ability to utilize hydrocarbon substrates is exhibited by a wide variety of bacterial and fungal genera. However, in the

marine environment, bacteria are generally considered to represent the predominant hydrocarbon-degrading element of the microbial community.

3.4.5 Relevance of the community to hypoxia and denitrification

This study adds to the knowledge of culturable heterotrophic bacteria along the WCI in terms of their community variations occurring in a spatiotemporal scale. This study shows that the bacterial community structure of coastal hypoxic ecosystems is phylogenetically quite diverse and distinct spatiotemporally. The ability of bacteria to respond to seasonal variations could allow them to adapt efficiently to changing environmental conditions. Many of the strains are reported as denitrifiers in the terrestrial environments. The high diversity of culturable bacteria in low oxygen conditions reflect their important ecological role in biogeochemical cycling in the coastal ecosystem. The bacterial community structure seems to be significantly impacted by the environmental variables which experience monsoon induced hydrographic changes. Indeed, as reflected by the rarefaction curves tending to plateau during all three seasons, the coverage of culturable diversity is quite adequate.

3.4.6 Conclusion

The presence of hydrocarbons as well as the organic matter produced in the WCI in response to upwelling and convective overturning is degraded by the bacteria found in the study area, the shortage of oxygen that follows forces these microbes to utilize nitrate to sustain growth thereby leading to denitrification. This explains that bacteria can adapt themselves well with the changing environment. There was no consistency in the diversity of marine bacterial populations and in their spatiotemporal distribution pattern. This may be due

to the physicochemical parameters that affect the occurrence and diversity of marine bacteria. The temporal changes in the bacterial population are found to be more pronounced in the coastal environment than the Open Ocean or deep sea (Chandrika and Nair, 1994; Jain et al. 2014). Statistical analyses showed that diversity was in the order of Goa>Mangalore>Kochi. This might be due to the bacterial preferences in accordance to their habitat choice or on the physicochemical and nutritional requirements. Low diversity during SuM may be attributed to the rainfall in the study area, as it is reported that bacterial dormancy is induced by the adverse environmental conditions (Lennon and Jones, 2011). Studies have reported that alterations in environmental conditions are important drivers of changes in microbial diversity (Castro et al. 2016). Murray et al. (1998) confirmed that stratification may result in more pronounced differences between different depths. The observations of this first work on the phylogenetics of culturable bacteria in the WCI experiencing seasonal hypoxia are useful to not only highlight the existence of plentitude of Phylogenetic diversity but also its dynamic shift during different seasons.

DENITRIFICATION POTENTIAL OF A SELECTED SET OF HETEROTROPHIC BACTERIAL CULTURES

4.1 <u>Introduction</u>

The biogeochemical transformation of biologically essential elements is performed by diverse microbial communities. Among such functions, the ability to denitrify (removal of fixed nitrogen) occurs widely in many taxonomic groups of bacteria (Braker et al. 2000; Jayakumar et al. 2004). Fixed nitrogen is used as an alternative electron acceptor to support respiration when oxygen is limiting. Denitrifiers are heterotrophic bacteria (most of them are facultative anaerobic) (Zumft, 1997; Yu et al. 2014) that pair the oxidation of organic substrates to the reduction of NO_3^- to either N_2O or N_2; not necessarily under strict anaerobic condition. Most bacteria can switch between oxic and nitrate dependent modes of respiration. The nitrous oxide produced in the process in turn yield a highly reactive free oxygen radical producing nitrogen oxides (NO_X) which is involved in stratospheric ozone depletion (Portmann et al. 2012) and global warming.

Besides its impact on the nitrogen cycle, a very important role of conventional denitrification is the degradation of organic carbon in the deficiency of oxygen. It was believed that denitrification would not occur in the presence of oxygen since there seems to be no energetic advantage to using nitrate as an oxidant when oxygen is accessible (Goreau et al.1980; Zehr et al. 2002; Jayakumar et al. 2009b). Experiments have since proven that denitrifiers are often facultative anaerobes (Schlesinger, 1997). Denitrifying species have been isolated from canals, ponds, soils, and activated sludge that can simultaneously utilize oxygen and nitrate as electron acceptors. These include *Paracoccus* (Lukow and Diekmann, 1997), *Pseudomonas* (Kesserű et al. 2003), *Bacillus* (Kim et al. 2005b), *Alcaligenes* (Robertson and Kuenen, 1983) etc.

The response and adaptability of microbial communities to climate change and other environmental change is an important component of ecosystem response, and several studies have investigated the response of denitrifiers to environmental change (Fenn et al. 1998; Erisman and Vries, 2000; Barnard et al. 2005). Due to the acknowledged significance of the Arabian Sea in the fixed nitrogen loss, and the reported intense denitrification in the eastern Arabian Sea (Jayakumar et al. 2004, Naqvi et al. 2000, 2006), it is important to study the contribution of bacterial communities to denitrification. This chapter focused on the selected set of culturable fractions involved in the utilization of transformation of nitrogen species in the environment.

4.2 Materials and Methods

4.2.1 Nitrate reduction test

To check the ability of nitrate reduction, all the cultures obtained in Chapter 3, checked for their capability to reduce nitrate to nitrite (or beyond) according to the protocol by Clarke and Cowan (1952). Briefly, th*e cultures were* grown in nitrate broth consisting of (5g peptic digest animal tissue, 3 g meat extract, 1 g KNO_3, 30 g NaCl per litre and final pH 7.6) for 24 hours, and checked for nitrate reduction by adding a few drops of Griess reagents (sulfanilic acid and α-naphthylamine). Nitrate reduction was indicated by broth forming pink and no change in color on addition of Griess reagents indicated no reduction of nitrate or reduction beyond nitrite. To check if nitrate is either reduced beyond nitrite or not at all, a pinch of zinc dust added to those tubes in which there was no color change after addition of Griess reagents. Broth turning to red after adding zinc dust was taken to indicate no reduction of nitrate.

From this test, out of 900 cultures, 673 were found to be nitrate reducers that further grouped into 42 different species and these 42 representatives were chosen for further analysis.

4.2.2 Determination of nitrate and nitrite

Preparation of reagents: Grasshoff et al. (1983)

1. *Ammonium chloride* (NH_4Cl) *buffer:* 10g ammonium chloride was dissolved in 1 L of distilled water and the pH adjusted to 8.5 with 1.5 mL of 25 % ammonia solution.

2. *Sulphanilamide solution*: 10g sulphanilamide was dissolved in 100mL C. HCl and 500mL of distilled water and made up to 1 L.

3. *N-(1-naphthy1)-ethylenediamine dihydrochloride* (*NED*): 0.5 g of the amine dihydrochloride was dissolved in 500mL of distilled water.

4. *Reductor filling:* Commercially available granulated cadmium (e.g., Merck) was sieved and the fraction between 40 and 60 mesh was retained and used.

5. *Copper sulphate* ($CuSO_4$): 1g of copper sulphate pentahydrate ($CuS0_4.SH_20i$) was dissolved in 100 mL of distilled water.

6. *Nitrate standard solution:* 1.011 g of dry potassium nitrate ($KN0_3$) was dissolved and made up to 1 L with distilled water. The stock solution contains 10 mmol/L nitrate.

7. *Nitrite standard solution:* Anhydrous sodium nitrite ($NaN0_2$) was dried at 100 ℃ for 1 h and 0.690 g is dissolved in 1 L of distilled water. The stock solution contains 10mmol/L nitrite.

8. *Nitrate broth*: 5 g peptic digest of animal tissue, 3 g meat extract, 1 g KNO_3, 30g NaCl was added to 1 L of distilled water at pH 7.

The cell-free extracts of cultures positive for the nitrate reduction test were further analyzed. The cell-free extracts were then determined for nitrate and nitrite as described by Grasshoff et al. (1983).

For the determination of nitrate, the cadmium granules were washed with 5% HCl and subsequently with distilled water until the pH was adjusted to 7. The granules were incubated overnight in 2% $CuSo_4$ and washed several times with NH_4Cl buffer. A small glass wool plug was inserted into the bottom of reduction column of 20 cm long and 4 mm diameter and filled with distilled water, followed by gently adding the Copper-Cadmium granules. A glass wool plug was inserted in the upper end of the tube and the column was rinsed thoroughly with NH_4Cl buffer. 0.5 ml of the cell-free extract of individual cultures were then made up to 50 ml volume with distilled water and passed through the reductor column allowing flow rates of about 7 ml/min. The first 25 ml was used to flush the reductor and the remaining 25 ml collected. 1 ml sulphanilamide was added to the sample and mixed. Subsequently, 1 ml of NED was added and allowed to react for 15 min and the absorbance was measured spectrophotometrically at 540 nm. The nitrate was reduced to nitrite in the Cd column and the total nitrite (nitrate plus nitrite) was determined spectrophotometrically and substituting the values in the standard graph.

Nitrite was determined by reaction with sulfanilamide and NED to 0.5 ml of the cell-free extract of individual cultures made up to 50 ml and the measurement of absorption at 540 nm and substituting the values in the standard graph.

4.2.3 Estimation of nitrate reduction rate (NRR)

In addition to testing the nitrate reduction in nitrate broth, 42 representative nitrate reducing cultures listed in Table 4.1 recognized through phylogenetic analyses were grown in Zobell marine broth for 24 hours and 5 µl each of these 42 cultures was added into nitrate broth. The cultures were grown in nitrate broth for 24 hours and denitrification rate was calculated as follows:

NRR=A-B/V X 24

NRR= Nitrate reduction rate day^{-1}

A= Initial concentration of NO_3 in broth

B= Final concentration of NO_3 in broth

V=Volume of broth

4.2.4 Quantification of nitrous oxide production

To quantify the nitrous oxide production, the 42 representative nitrate reducing cultures were grown in nitrate broth for 24 hours and their growth stopped by adding 0.3 ml saturated mercuric chloride. The cultures were centrifuged at 12000 rpm for 10 min to collect the supernatant for measuring N_2O production as described by Sudheesh et al. (2016). Briefly, 25 ml of sample was equilibrated successively with an equal volume of ultrapure helium in an air-tight syringe by vigorously shaking the syringe at room temperature for 5 min using a wrist action shaker. After attaining equilibrium, the gas mixture from the headspace was injected through a 5 ml sampling loop into a gas chromatograph (Shimadzu) and separated over a Chromosorb column (80/100 mesh) at 40°C. The detection of N_2O was done in a 63Ni Electron Capture Detector (ECD). The Precision of the analysis was ~4%. Calibration was

achieved using a gas mixture of N_2O in nitrogen (Alltech Associated Inc., IL, USA). The percentage saturation of N_2O was calculated as

N_2O saturation= (C_w/C_a) x 100

Where, C_w is the measured concentration of N_2O and

C_a is the equilibrium concentration

The per unit OD 600 and per unit cell of NRR and nitrous oxide produced was calculated

NRR per unit OD 600 = NRR μM day $^{-1}$ / OD 600

N_2O per unit OD 600 = N_2O nM day $^{-1}$ / OD 600

NRR 10^{-14} mol cell $^{-1}$ = NRR μM day $^{-1}$ / Viable counts

4.2.5 Nitrate reductase enzyme assay

Nitrate reductase activity in cell-free extracts from all 42 representative nitrate reducing cultures was assayed by Smarrelli and Campbell (1983), Redinbaugh and Campbell (1985). The reaction mixture for nitrate reductase (final volume, 2 ml) contained 24 mM potassium phosphate buffer with 9.5 mM potassium nitrate and 0.05 mM EDTA at pH 7.3, and 100 μl of the supernatant that contained the enzyme β-NADH(electron donor). The nitrite produced was determined by adding 1 ml each of diazo coupling reagent (58mM sulfanilamide in 3M HCl solution and 0.77 mM N-(1-napthyl)-ethylenediamine) and absorbance was measured at 540 nm. The enzyme activity was deduced by drawing the standard graph of nitrite prepared by using known concentrations of nitrite. Nitrate reductase activities expressed as U ml^{-1}. One unit of NR activity is defined as 1 μmol of nitrite produced per min at pH 7.3 at 30°C.

4.2.6 PCR amplification of *narG*, *nirS* and *nosZ* gene and sequencing

PCR amplification of denitrifying genes was performed in 50 µl volume using Taq PCR reaction mix as per manufacturer's instructions (Sigma-Aldrich). Amplicons of *narG* and *nirS* and were obtained according to the protocol of Bru et al. (2007) and Throbäck et al. (2004). After the initial denaturation at 95 °C for 10 min, 6 cycles of 95 °C for 15 s, 63 °C for 30 s, and 72 °C for 30 s of touchdown was carried out, with a 1 °C step down in annealing temperature of each cycle. Followed by 35 cycles of 95 °C for 15 s, 58 °C for 30 s, 72 °C for 90 s and a final extension at 72 °C for 10 min, the *nosZ* gene was amplified using the primer set NosZ1840-F and NosZ2090-R (Henry et al. 2006). After an initial denaturing step at 95 °C for 10 min, 6 cycles of 95 °C for 15 s, 65 °C for 30 s, and 72 °C for 30 s of touchdown was carried out, with a 1°C step down in annealing temperature of each cycle. This was followed by 35 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 90 s and a final extension at 72 °C for 10 min. The PCR products were observed on 1.5 % (w/v) ethidium bromide-stained agarose gel to ensure that the correct size fragment were amplified. The purified PCR products were then sequenced by using an ABI 3130XL genetic analyzer (Applied Bio-Systems). Sequences were checked for their similarity with sequences in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov).

4.3 Results

4.3.1 Nitrate reduction test and nitrate reduction rate (NRR)

Of the 900 cultures obtained from the study area as described in chapter 3, 673 cultures were nitrate reducers accounting for 75% of the total bacterial population in this

study. Out of these, 42 different individual cultures representing the 673 nitrate reducers were obtained. The 42 representative nitrate reducers were chosen for further analysis.

NRR ranged from 0.04–0.27 μMd^{-1} among the 42 cultures. The per cell NRR ranged from 0.41-2.40 (X 10^{-14}) mol cell^{-1}. It was seen that *Halomonas axialensis* recorded the highest NRR of 2.40 (X 10^{-14}) mol cell^{-1}. NRR was the least [0.41-0.57 (X 10^{-14}) mol cell^{-1}] by *Salinicola salarius* and *Microbacterium aquimaris* (Table 4.1).

4.3.2 Nitrous oxide production from the bacterial cultures

Selected 42 representative nitrate reducing cultures were checked for nitrous oxide (N_2O) production. N_2O production ranged from 7.00-119.32 nMd^{-1} among all 42 cultures. The per cell N_2O ranged from 0.29-5.54 (X 10^{-15}) mole cell^{-1}. Maximum N_2O production was by *Idiomarina sediminium* and *Marinobacter salsuginis* in the range of 5.18-5.54 (X 10^{-15}) mole cell^{-1}, followed by *Halomonas axialensis* [3.36 (X 10^{-15}) mole) cell^{-1}]. *Bacillus aryabhattai* produced the least N_2O [0.29 (X 10^{-15}) mole cell^{-1}] (Table 4.2).

4.3.3 Nitrate reductase enzyme activity assay

The nitrate reductase activities ranged among the tested cultures (Table 4.1) was low as 0.012-0.037 UmI^{-1} by *Micrococcus endophyticus, Vibrio tubiashii* and *Bacillus vietnamensis*. Notably, the highest activity of 0.084 and 0.119 U ml^{-1} was by *Idiomarina sediminium* and *Halomonas xianhensis* respectively.

4.3.4 Nucleotide sequence accession numbers

Sequences for *nos*Z gene were submitted to GenBank and accession numbers assigned are MG948595-MG948604; MG958092-MG958111; MG983072-MG983083, MG997038-MG997079, MH025494-MH025535.

Table 4.1: Viable cell counts, optical density, nitrate reductase activity (NRA) and nitrate reduction rate (NRR) production by different bacterial cultures grown in nitrate broth

Sr. no.	Culture	Viable cells (No.X10^7ml^{-1})	OD $_{600}$	NRA (U ml^{-1})	NRR (μM d^{-1})	NRR (μM) per unit OD	NRR (10^{-14} mol cell^{-1})
1	*Salinicola salarius*	1.7	1.10	0.054	0.07	0.06	0.41
2	*Microbacterium aquimaris*	1.4	1.50	0.071	0.08	0.05	0.57
3	*Grimontia marina*	2.0	1.23	0.059	0.12	0.09	0.60
4	*Anaerobacillus alkalilacustris*	2.3	0.65	0.055	0.14	0.21	0.61
5	*Kocuria flava*	2.9	1.70	0.071	0.19	0.11	0.65
6	*Bacillus aryabhattai*	2.7	1.13	0.057	0.18	0.16	0.66
7	*Staphylococcus arlettae*	0.6	0.89	0.054	0.04	0.04	0.67
8	*Vibrio alginolyticus*	2.8	0.32	0.068	0.19	0.60	0.67
9	*Schwanella coralli*	1.6	1.25	0.072	0.11	0.08	0.68
10	*Marinobacter salsuginis*	2.1	1.60	0.070	0.16	0.10	0.70
11	*Staphylococcus haemolyticus*	0.7	0.92	0.053	0.05	0.05	0.71
12	*Vibrio fortis*	2.3	1.35	0.068	0.17	0.13	0.73
13	*Idiomarina seosinensis*	2.6	1.80	0.070	0.20	0.11	0.76
14	*Zunongwangia profunda*	2.9	0.75	0.068	0.23	0.31	0.79
15	*Marinomonas communis*	1.7	1.18	0.075	0.14	0.12	0.82
16	*Alteromonas macleodii*	2.8	1.77	0.070	0.23	0.13	0.82
17	*Janibacter melonis*	2.0	0.90	0.062	0.17	0.19	0.85
18	*Acinetobacter seohaensis*	1.4	1.52	0.061	0.12	0.07	0.85
19	*Sufflavibacter maritimus*	2.9	1.60	0.066	0.25	0.16	0.86
20	*Micrococcus endophyticus*	0.8	1.42	0.012	0.25	0.17	0.86
21	*Kosakonia cowanii*	2.6	1.30	0.072	0.23	0.18	0.88
22	*Psychrobacter maritimus*	2.7	1.30	0.054	0.24	0.18	0.88
23	*Halomonas xianhensis*	2.8	1.00	0.119	0.27	0.27	0.90
24	*Pseudomonas stutzeri*	2.8	1.60	0.072	0.27	0.17	0.90
25	*Bacillus cereus*	2.6	1.60	0.057	0.24	0.15	0.92
26	*Bacillus subtilis*	2.5	0.93	0.066	0.23	0.25	0.92
27	*Marinobacter hydrocarbonoclasticus*	2.9	1.50	0.074	0.27	0.18	0.93

Table 4.1 continues

Sr. no.	Culture	Viable cells (No.X10^7ml^{-1})	OD $_{600}$	NRA (U ml^{-1})	NRR (µM d^{-1})	NRR (µM) per unit OD	NRR (10^{-14} mol cell^{-1})
28	*Idiomarina zobellii*	2.1	0.90	0.066	0.20	0.22	0.95
29	*Idiomarina sediminium*	2.3	1.00	0.084	0.25	0.25	1.00
30	*Vibrio rotiferanus*	1.3	1.35	0.058	0.13	0.09	1.00
31	*Bacillus vietnamensis*	1.8	1.75	0.037	0.19	0.10	1.06
32	*Vibrio brasiliensis*	1.4	0.23	0.061	0.19	0.82	1.30
33	*Vibrio campbellii*	1.2	0.70	0.069	0.17	0.24	1.41
34	*Bacillus firmus*	1.3	0.53	0.073	0.20	0.38	1.53
35	*Bacillus circulans*	1.5	1.55	0.058	0.23	0.14	1.53
36	*Vibrio tubiashii*	1.1	1.22	0.025	0.18	0.15	1.64
37	*Thalassospira tepidiphila*	1.5	1.00	0.061	0.26	0.26	1.70
38	*Vibrio caribbeanicus*	0.8	0.36	0.062	0.14	0.39	1.70
39	*Chromohalobacter israelensis*	0.9	0.54	0.054	0.18	0.33	1.80
40	*Vibrio chagasii*	1.1	1.56	0.062	0.24	0.15	2.18
41	*Marinobacter gudaonensis*	1.0	1.48	0.075	0.22	0.15	2.20
42	*Halomonas axialensis*	0.9	0.66	0.068	0.24	0.36	2.40

Table 4.2: Nitrous oxide (N_2O) production by different bacterial cultures grown in nitrate broth

Sr. no.	Culture	N_2O (nM d^{-1})	N_2O (nM) per unit OD	N_2O (10^{-15} mol cell^{-1})
1	*Bacillus aryabhattai*	7.92	7.00	0.29
2	*Staphylococcus arlettae*	5.54	6.22	0.05
3	*Bacillus vietnamensis*	6.23	3.56	0.34
4	*Grimontia marina*	7.22	5.86	0.36
5	*Anaerobacillus alkalilacustris*	8.56	13.16	0.37
6	*Staphylococcus haemolyticus*	4.95	5.38	0.38
7	*Schwanella coralli*	6.23	4.98	0.38
8	*Vibrio alginolyticus*	11.47	35.84	0.40
9	*Pseudomonas stutzeri*	11.73	7.33	0.41
10	*Psychrobacter maritimus*	12.35	9.50	0.45
11	*Acinetobacter seohaensis*	6.32	4.15	0.45
12	*Kosakonia cowanii*	12.20	9.38	0.46
13	*Alteromonas macleodii*	13.84	7.81	0.41
14	*Bacillus cereus*	13.51	8.44	0.51
15	*Vibrio fortis*	12.18	9.02	0.52
16	*Idiomarina seosinensis*	14.52	8.06	0.55
17	*Janibacter melonis*	11.65	12.94	0.58
18	*Kocuria flava*	13.48	7.92	0.64
19	*Micrococcus endophyticus*	5.25	3.69	0.65
20	*Bacillus circulans*	11.21	7.23	0.74
21	*Salinicola salarius*	13.36	12.14	0.78
22	*Idiomarina zobellii*	16.91	18.78	0.80
23	*Microbacterium aquimaris*	11.63	7.75	0.83
24	*Vibrio brasiliensis*	12.84	55.82	0.91
25	*Marinobacter hydrocarbonoclasticus*	29.28	19.52	1.00
26	*Halomonas xianhensis*	28.91	28.91	1.06
27	*Bacillus subtilis*	28.72	30.88	1.14
28	*Vibrio chagasii*	12.79	8.19	1.16
29	*Zunongwangia profunda*	35.98	47.97	1.24
30	*Sufflavibacter maritimus*	38.38	23.98	1.32
31	*Chromohalobacter israelensis*	12.89	23.87	1.43
32	*Vibrio caribbeanicus*	12.35	34.30	1.45
33	*Vibrio rotiferanus*	11.24	8.32	1.60
34	*Vibrio tubiashii*	10.21	8.36	1.70
35	*Marinomonas communis*	30.19	25.58	1.77
36	*Marinobacter gudaonensis*	22.11	14.93	2.20
37	*Vibrio campbellii*	27.22	38.88	2.26
38	*Bacillus firmus*	30.32	57.20	2.33
39	*Thalassospira tepidiphila*	35.48	35.48	2.36
40	*Halomonas axialensis*	30.30	45.90	3.36
41	*Idiomarina sediminium*	119.32	119.32	5.18
42	*Marinobacter salsuginis*	116.37	72.73	5.54

4.4 <u>Discussion</u>

The most important question to be addressed in microbial ecology is which members of bacterial communities are responsible for the overall activity, and what factors control the activity or inactivity, of *in situ* populations. Culture-based studies provide information on the physiological characteristics of the organisms and their activity to the functioning of this ecosystem. Heterotrophic bacteria play a significant role in the biogeochemical cycling in the ocean because of their high abundance and ubiquity. In the study area, both aerobic and facultative anaerobic heterotrophic denitrifying bacteria apparently are important functional groups in the nitrogen cycle, due to their ability to respire anaerobically using nitrogen oxides as electron acceptors, which are reduced to nitrous oxide and dinitrogen (Gomes et al. 2017).

Denitrifiers are mostly heterotrophic, capable of, and perhaps preferring, aerobic respiration, although the thermodynamic yield of growth using nitrate compares quite analogously to that of oxygen (Ward et al. 2007). As mentioned earlier Kesserű et al. (2003), Kim et al. (2005b) and Li et al. (2013) have isolated aerobic denitrifying species from canals, ponds, soils, activated sludge that are known to simultaneously utilize oxygen and nitrate as electron acceptors. Though this appears to contradict the fact that denitrification is an anaerobic process, these reports effectively demonstrate proving that denitrification is not necessarily or exclusively anaerobic. An important role of bacterial denitrification, in addition to its impact on the nitrogen cycle, is the degradation of organic carbon in deoxygenated oceanic realms, as Ward et al. (2007) observed when obligately aerobic heterotrophs cannot function.

Metabolically versatile heterotrophic bacterial assemblages adapted to seasonally fluctuating oxygen concentrations are very likely to perform a great deal of nitrate respiration

which, with its very high oxidation state, is an ideal electron acceptor. Thus, nitrate reduction is an ecologically vital process carried out by phylogenetically diverse bacteria (Lavermann, 2006; Correa-Galeote et al. 2013). While there are no earlier experimental data on NRR from individual bacterial cultures, reports of sedimentary NRR in the range of 0.03 to 1.5 μM cm^{-3} h^{-1} (Hordijk et al. 1987) and 0.274–0.933 μM cm^{-3} h^{-1} (Laverman et al. 2006). Naik and Naqvi (2002) reported nitrite formation rates of 0.17–1.33 mmol N m^{-2} d^{-1} from the Arabian Sea continental shelf along the west coast of India.

Nitrous oxide production was demonstrated to be a very reliable and reproducible feature of the cultures. The highest concentration of *in situ* nitrous oxide in the study area recorded earlier by Naqvi et al. (2006) was 765 nm. However, this study on N_2O production from culturable bacteria might come handy to work out the global marine N_2O production from the well known denitrifying oxygen minimum zones, despite the large differences in N_2O production by different tested cultures. For instance, one of the most active denitrifying heterotrophic bacterium *Pseudomonas stutzeri*, is considered as a model organism for studying denitrification process (Zumft, 1997; Lalucat, 2006). Takaya et al. (2003) reported N_2O production from *Pseudomonas stutzeri* to be in the range of 1.5 x 10^{-2} to 6.75 x 10^{-5} (μmol min^{-1} OD_{540}^{-1}), whereas the same bacterium in our study produced N_2O of 7.81 nM OD_{600}^{-1}. Both *Marinobacter hydrocarbonoclasticus* (Li et al. 2013) and many different species of *Bacillus* (Verbaendert et al. 2011) are reported to be efficient denitrifiers. Assays involving nitrate reductases of marine bacteria are useful to note rates of nitrate utilization. The first step in denitrification is the reduction of nitrate to nitrite is catalyzed by nitrate reductase. Nitrate reductase assay from the marine origin was earlier reported in phytoplankton (Hurd et al. 1995) and microalgae (Chow et al. 2007).

Results from this study prove useful in recognizing the contribution to denitrification by culture amenable heterotrophic bacterial species examined. It is worthwhile to note from our study that the rate of nitrate reduction/nitrite formation/nitrous oxide production is quite different among the strains we tested. Most of these facultative anaerobes grew on Zobell medium aerobically implying that the ability of nitrate reduction is widely distributed among the culture amenable bacterial genera. Another result worthy to note from this study is the observed wide difference both in NRR and N_2O production amongst the representatives of 42 different denitrifying species these denitrifying coastal waters. The 42 representative species were also checked for the denitrification genes and the accession numbers were obtained.

Conclusion

The utilization and transformation of nitrate to nitrous oxide by many bacterial cultures indicate their potential role in the nitrogen cycle in the coastal regions experiencing temporal differences in dissolved oxygen concentrations sometimes reaching hypoxic levels. Diverse and high percentage of culturable bacteria reducing nitrate in low oxygen conditions reflect their important ecological role in denitrification processes in the coastal ecosystem. The efforts of examining individual cultures capable of reducing nitrate to nitrite and examining their nitrate reduction rate (NRR), nitrous oxide (N_2O) production under laboratory conditions would prove useful in assessing the range of possible denitrification potential.

METAGENOMIC DNA ANALYSIS BASED BACTERIAL COMMUNITY STRUCTURE OFF GOA

5a.1 <u>Introduction</u>

Bacteria are composed of different taxonomic groups with potentially different phenotypic properties, physiological activities, and ecological functions although they are unicellular and similar in cell size. More interestingly, as Azam et al. (1983; 1994) stated bacterial community structure and environmental variables are the driving forces leading to specialized ecological functions bacteria perform within marine carbon and nutrient cycles.

Of the total bacterial diversity existing in nature, only a small fraction of bacteria can be brought into cultures in the laboratory. With the advent of molecular-based techniques, studies have focused on the diversity of microbial communities. The culture-based approach is useful for understanding the physiological potential of isolated organisms, but the drawback is that it does not provide the complete composition of microbial communities. The results obtained by culture-dependent techniques cover only those few organisms that could be cultivated. Various studies have employed culture-independent techniques to show that cultivated microorganisms from diverse environments often may represent a minor component of the microbial community as a whole. It is accepted that cultivation methods recover less than 1% of the total microorganisms present in environmental samples (Amann et al. 1995; Ward et al. 1990). Therefore, microbial investigations based only on cultivation strategies cannot be regarded as reliable in terms of reflecting the microbial diversity presents.

At all levels of bacterial phylogeny, uncultured clades are known to play critical roles in cycling carbon, nitrogen, and other elements, synthesizing novel natural products, and impacting the surrounding organisms and environment. Our ability to extract DNA from the environmental sample and obtain sequence information by PCR amplification followed by cloning or direct sequencing allow classification of bacteria on the basis of 16S rRNA gene

sequences, irrespective of organism's viability. Through such analyses, a hidden 'ocean of diversity' that had never been seen by cultivation (Amann et al. 1995; Stewart, 2012) is revealed.

5a.2 <u>Materials and methods</u>

5.2.1 Sample Collection

Details on the location of the study area are provided in Chapter 3 (Section 3.2.2). For phylogenetic analyses, seawater samples collected from G9 (Off Goa) during all three seasons were used.

5a.2.2 Extraction of Metagenomic DNA

Volumes of 2.5 L of seawater from each depth were passed peristaltically through Sterivex cartridge fitted with 0.22 μm pore size membrane filter (Millipore, USA) to retain microbial cells for DNA extraction. The cartridges were then filled with 1.8 ml lysis buffer (Tris pH 8.3, 40 mM EDTA and 0.75 M Sucrose), sealed and stored at -80 °C until DNA extraction was carried out in the laboratory following Ferrari and Hollibaugh (1999). 40 μl of lysozyme (50 mg ml^{-1}) was added to each cartridge and then incubated for 60 min at 37 °C. 100 μl of proteinase K (20 mg ml^{-1}) and 100 μl of sodium dodecyl sulfate (SDS; 20 % wt/vol) were added to each cartridge, and the cartridges were further incubated at 55 °C for 2 hours. The DNA from the lysate was purified by sequential extraction with equal volumes (2 ml) of phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1) by centrifuging at 12000 rpm for 10 min. The aqueous phase was separated and precipitated overnight at -20 °C with 500 μl isopropanol and centrifuged at 12000 rpm for 15 min. The precipitated DNA was washed with 70 % chilled ethanol. It was finally dissolved in 40 μl TE

buffer (10 mM Tris and 1 mM EDTA, pH 8.0) and its integrity was checked by agarose (0.8 %) gel electrophoresis.

5a.2.3 PCR amplification of 16S rRNA gene from DNA extracts

Details of PCR amplification is described in section 3.2.7.

5a.2.4 Clone library construction

PCR products were purified using UltraClean® PCR Clean-Up Kit. The PCR product was ligated into a pCR® 4-TOPO plasmid vector and cloned into TOP10 One Shot competent *Escherichia coli* cells as per manufacturer's instructions (Invitrogen). The cloning reaction consisted of 2 µl of purified PCR product, 1 µl of Salt solution (1.2 M NaCl; 0.06 M MgCl$_2$), 2 µl of sterile distilled water and 1 µl of TOPO® vector. The reaction mixture was incubated for 30 min on ice and 3 µl of the reaction mixture was added to a vial of competent cells for 30 min. The cells were given heat shock at 42 °C for 30 seconds without shaking and immediately transferred to ice. After 5 min of incubation on ice, 250 µl of sterile S.O.C medium was added to this mixture and shaken horizontally (200 rpm) at 37 °C for 1 h and plated on LB plates containing X-Gal and incubated at 37 °C for 24 hours. Transformants were selected on Luria-Bertani agar plates containing ampicillin (100 µg ml^{-1}) and X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) (20 µg ml^{-1}). White colonies were picked up from each sample and screened by PCR using the vector primers M13F (5' GTAAA ACGAC GGCCA GT3') and M13R (5' CAGGA AACAG CTATG AC3'). The amplification was performed as follows: 10 min initial heating for 95 °C, 35 cycles each of 1 min at 94 °C, at 55 °C and at 72 °C and, a final 10 min extension step at 72 °C. The PCR products were

electrophoresed in 1% agarose gel for checking and selecting the positive clones with DNA inserts.

5a.2.5 DNA sequencing, phylogenetic tree construction and statistical analysis

The PCR products were purified and sequenced using ABI 3130XL genetic analyzer (Applied Bio-Systems) available in-house. Obtained partial sequences were trimmed using DNA baser software version 3.0 and vectors were removed using NCBI online program VecScreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/) and checked for the chimera using DECIPHER's (http://decipher.cee.wisc.edu/). Sequences were searched for similarity and compared with the NCBI database through BLAST searches (http://blast.ncbi.nlm.nih.gov). Sequences were grouped as operational taxonomic units (OTUs) by 97 % or greater sequence similarity, using the MOTHUR program (Schloss et al. 2009). Representative sequences from each OTU were aligned using the software MUSCLE (Edgar, 2004), and phylogenetic trees were constructed by the software MEGA6 using the neighbor-joining algorithm (Tamura et al. 2013). Bootstrap analysis was conducted using 1000 replicates. Rarefaction analysis and diversity indices (Shannon and Simpson), non-parametric diversity index (Chao I) as well as coverage for each season and depths were calculated using MOTHUR at the cut-off level of 3% using Mothur's summary.single routine.

5a.2.6 Nucleotide sequence accession numbers

Sequences were submitted to GenBank and accession numbers assigned are KX179919 to KX179927, KP076438 to KP076559, KT361318 to KT361395, KU498049 to KU498248, KX179928 to KX180027, KX238959 to KX260224, MF480560 to MF480649, MF540159 to MF540248 and MF588563 to MF588652.

5a.3 Results

5a.3.1 Bacterial community structure Off Goa

A total of 870 clones, at least 290 each from each season and 96 from each sampling depth were subjected to 16S rRNA gene sequencing. The sequences so obtained could be categorized into 14 lineages: *Gammaproteobacteria*, *Alphaproteobacteria*, *Cyanobacteria*, *Deltaproteobacteria*, *Firmicutes*, *Betaproteobacteria*, *Acidobacteria*, *Actinobacteria*, *Marinimicrobia*, *Verrucomicrobia*, *Bacteroidetes*, *Planctomycetes*, *Chloroflexi* and *Omnitrophica bacterium*.

5a.3.1.1 Seasonal variations in bacterial community

Seasonal comparisons were useful to make out that the bacteria were diverse during FIM followed by SIM and less diverse during SuM. *Proteobacteria* was the major phylum extant in all the seasons. *Cyanobacteria, Acidobacteria, Actinobacteria, Marinimicrobia* and *Verrucomicrobia* were also found to be consistently present during all three seasons. Clones of *Cyanobacteria* were the most abundant in the oxygenated surface layers during all three seasons. Clones of *Deltaproteobacteria* were found mainly during FIM and SuM. *Bacteroidetes* and *Firmicutes* were observed during FIM and SIM. *Planctomycetes, Chloroflexi* and *Omnitrophica bacterium* were found exclusively during FIM.

5a.3.1.2 Major Phylogenetic groups during SIM

Ten different Phylogenetic groups were obtained during SIM. *Gammaproteobacteria* was the dominant group observed during SIM, followed by *Cyanobacteria*. Pronounced vertical difference in the structure of bacterial community was observed. *Cyanobacteria* and *Betaproteobacteria* were found at the oxygenated surface, while *Gammaproteobacteria*, *Alphaproteobacteria* and *Actinobacteria* were observed throughout the water column. Minor

groups of bacteria like *Firmicutes, Bacteroidetes, Marinimicrobia, Acidobacteria* and *Verrucomicrobia* were observed at sub-surface waters (Fig. 5a.1a). The bacterial groups increased vertically from surface to bottom depth.

5a.3.1.3 Major Phylogenetic groups during SuM

As seen in SIM, *Cyanobacteria* and *Betaproteobacteria* was also observed during SuM with major dominant groups being *Gammaproteobacteria* and *Cyanobacteria*. *Gammaproteobacteria* was found to be increasing towards the bottom. *Alphaproteobacteria* was more prominent at the mid layer. The relative abundance of *Alphaproteobacteria* substantially decreased towards the bottom with the increase of Gamm*aproteobacteria* with water depth. Minor groups of bacteria like *Deltaproteobacteria Marinimicrobia, Acidobacteria, Actinobacteria* and *Verrucomicrobia* were observed at the bottom layer (Fig. 5a.1a).

5a.3.1.4 Major Phylogenetic groups during FIM

As compared to SIM and SuM, FIM witnessed more number of bacterial groups. 14 different Phylogenetic groups were obtained during SIM. *Planctomycetes, Chloroflexi* and *Omnitrophica bacterium* were evidenced during FIM *Gammaproteobacteria* was once again observed to the dominant group, followed by *Alphaproteobacteria*. Unlike SuM, *Deltaproteobacteria* was found to be increasing towards the bottom. Unlike SIM and SuM, the minor groups of bacteria like *Acidobacteria*, *Betaproteobacteria*, *Actinobacteria* and *Marinimicrobia* were found all through the water column, with the exception of *Chloroflexi* and *Firmicutes* being found at the mid layers (Fig. 5a.1a).
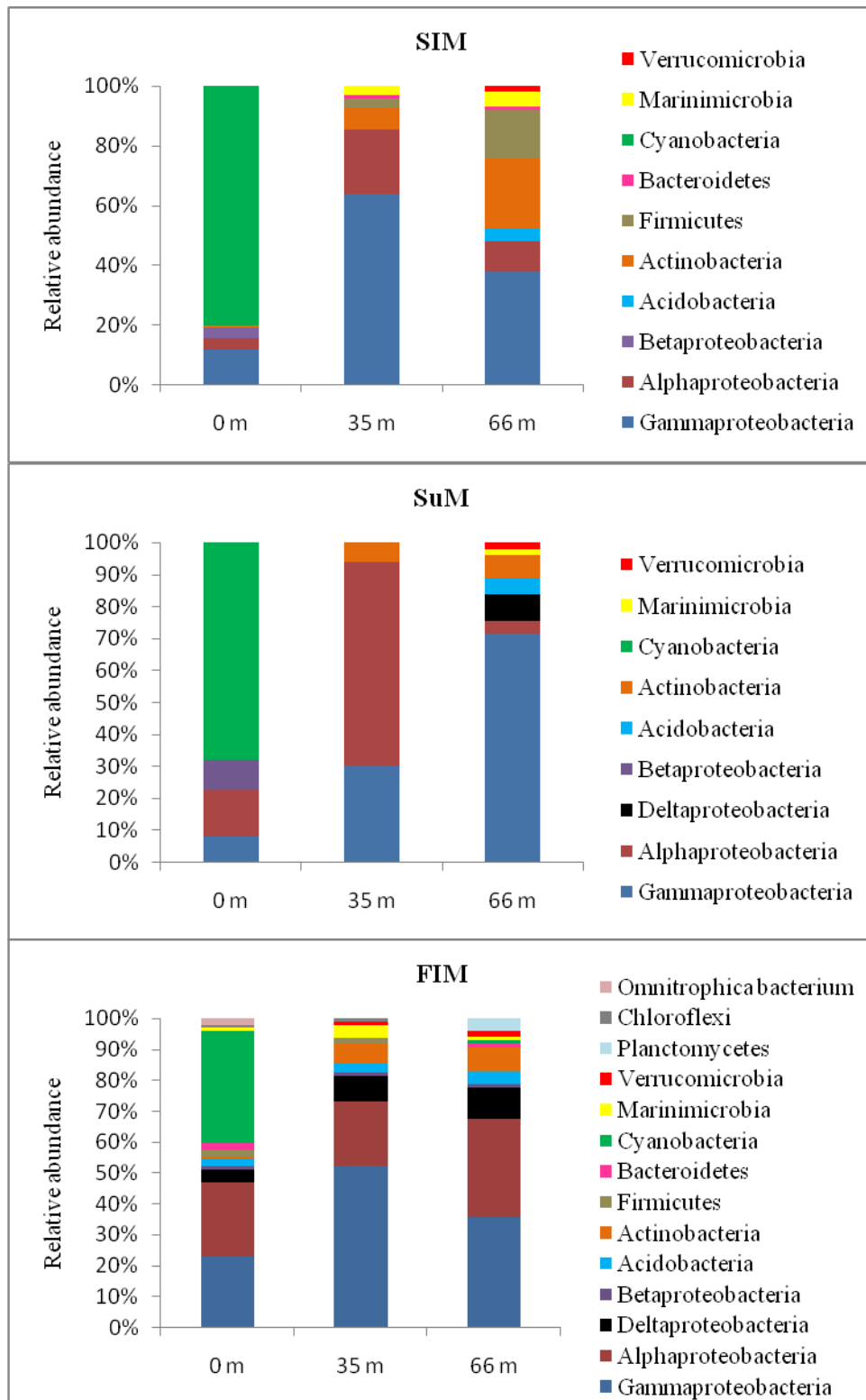
Fig.5a.1a: Bacterial community structure during different seasons off Goa

5a.3.2 Phylogenetic analysis of bacterial 16SrRNA genes across all three seasons

Phylogenetic trees were built to show relationships between the dominant OTUs in each season and their closest neighbors taken after grouping with MOTHUR.

5a.3.2.1 SIM: As many as 86 OTUs (or phylogroups) formed from the 290 clones, of which, 28 OTUs (109 clones) represented *Gammaproteobacteria* affiliating to *Alteromonas*, *Colwellia*, *Vibrio*, SAR86 cluster, *Idiomarina*, *Oceanobacter*, *Halomonas*, *Marinobacter*, *Pseudomonas* sp. respectively and uncultured *Gammaproteobacteria*. (20 OTUs; 35 sequences) belonged to *Alphaproteobacteria* with clones affiliating to SAR11 cluster, *Rhodospirillales*, *Rhodobacteraceae* and uncultured *Alphaproteobacteria*, *Cyanobacteria* (21 OTUs; 77 sequences), *Firmicutes* (4 OTUs; 19 sequences) belonged to *Bacillus* sp., *Marinimicrobia* (3 OTUs; 8 sequences) belonging to *Deferribacteres* sp. and uncultured *Marinimicrobia* sp., *Betaproteobacteria* (1 OTU; 3 sequences) belonged to *Burkholderia* sp., *Actinobacteria* (3 OTUs; 31 sequences)*, Acidobacteria* (3 OTUs; 4 sequences)*, Verrucomicrobia* and *Bacteroidetes* (1 OTU; 2 sequences each) (Fig 5a.2a, 5a.2b and 5a.2c).
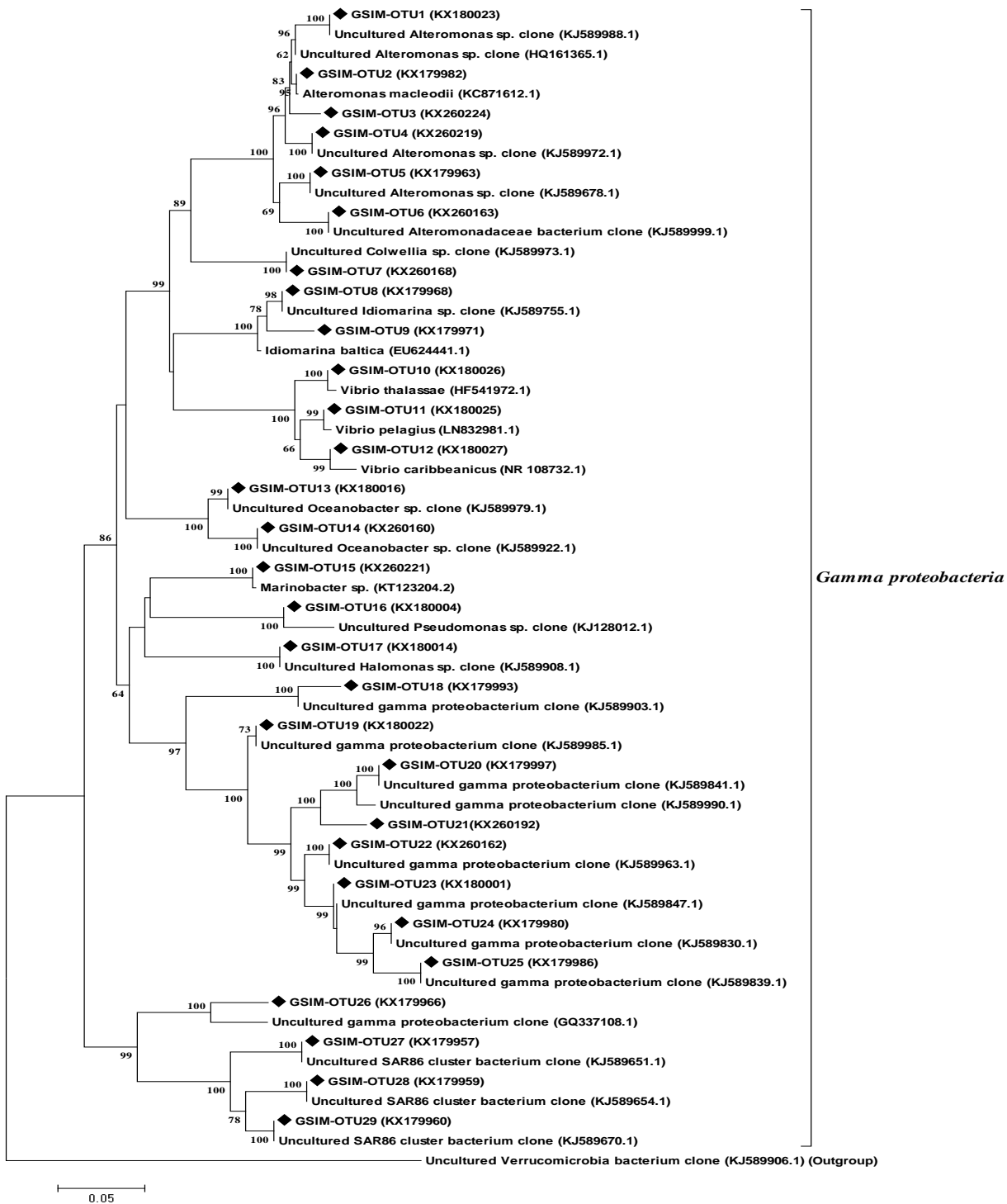
Fig 5a.2a
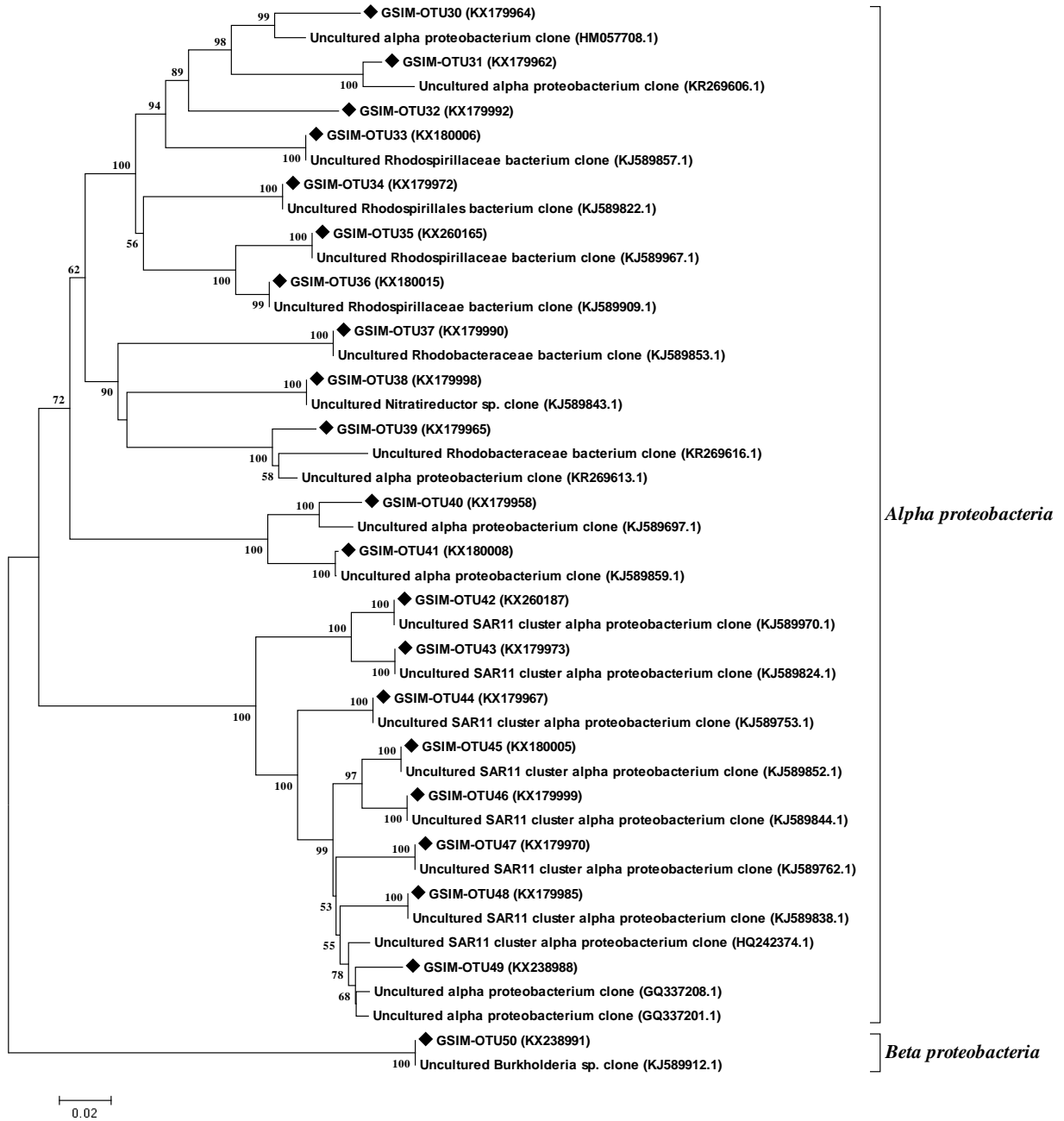
Fig 5a.2b
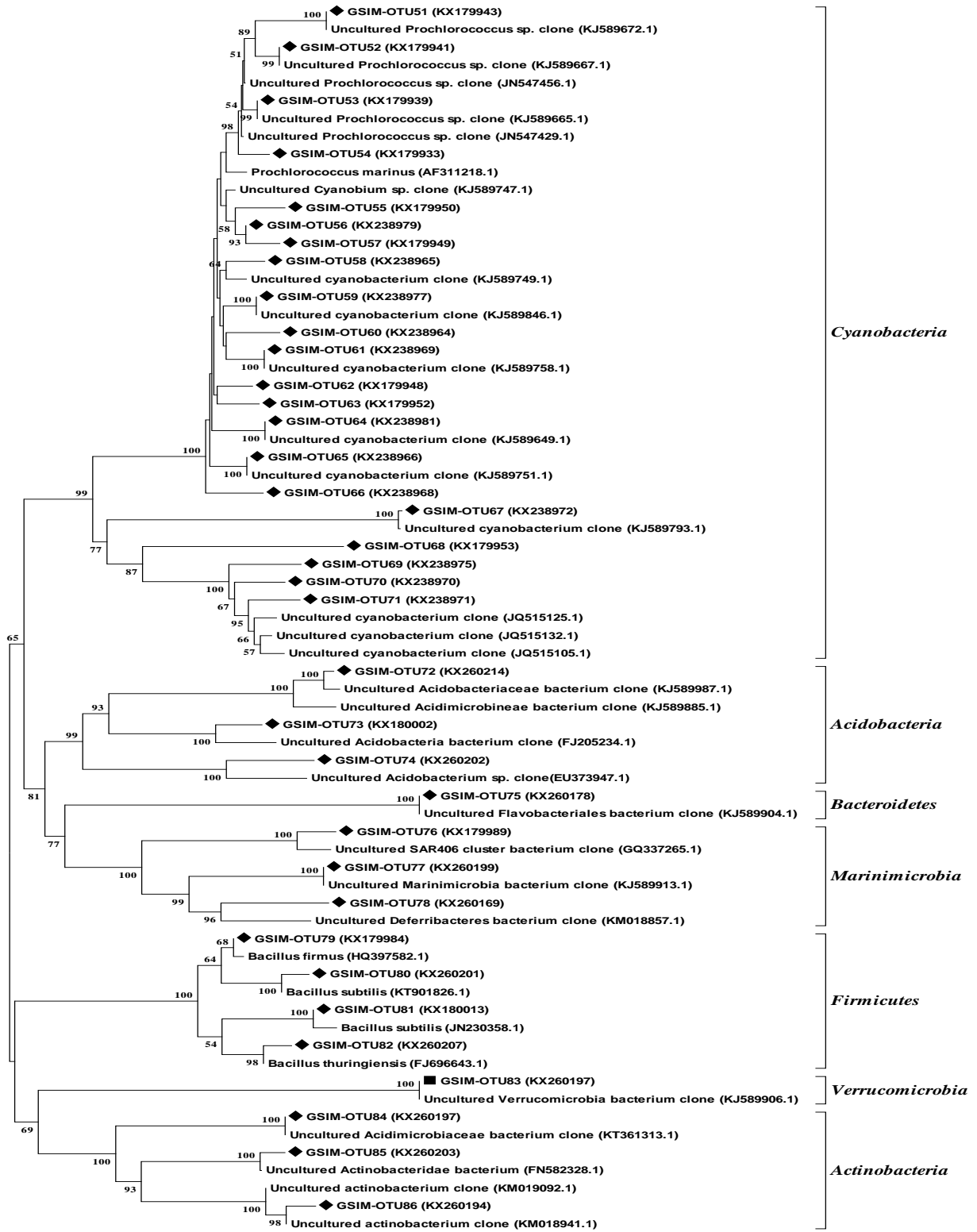
Fig 5a.2 c

Figure 5a.2: Neighbor joining tree of 16S rRNA gene sequences obtained from the water samples collected off Goa during SIM: Spring Inter-Monsoon, Sequences are aligned with the best matches available in the Genbank. Tree topology was inferred by using 1000 bootstrap iterations. Bootstrap values above 50% at each node. Scale bar represents the nucleotide substitution percentage.

a) Gammaproteobacteria
b) Alphaproteobacteria and Betaproteobacteria
c) Cyanobacteria, Acidobacteria, Bacteroidetes, Marinimicrobia, Firmicutes, Verrucomicrobia and Actinobacteria

5a.3.2.2 SuM: The 290 clones sequenced made up 73 OTUs. 33 OTUs (79 clones) belonged to *Alphaproteobacteria, w*ith clones affiliating to SAR11 cluster*, Marivita roseacus, Loktanella hongkongensis, Nitratireductor aquibiodomus, Rhodospirillaceae, Rhodobacteraceae, Erythrobacter*, *Sphingomonas, Donghicola, Roseobacter* sp. respectively and uncultured *alphaproteobacterium. Gammaproteobacteria* (18 OTUs; 107 sequences) belonged to *Oceanospirillaceae, Alteromonas,* SAR86 cluster*, Sinobacteraceae, Aestuariibacter*, *Thalassomonas*, *Vibrio*, *Oceanobacter* sp. respectively and uncultured *gamma proteobacterium, Betaproteobacteria* (2 OTUs; 9 sequences) belonged to *Burkholderia* sp., *Deltaproteobacteria* (6 OTUs; 8 sequences), *Actinobacteria* (4 OTUs; 13 sequences) and *Acidobacteria* (4 OTUs; 5 sequences), *Cyanobacteria* (3 OTUs; 65 sequences), *Marinimicrobia* (2 OTUs; 2 sequences) and *Verrucomicrobia* (1 OTU; 2 sequences) (Fig 5a.3a, 5a.3b and 5a.3c).
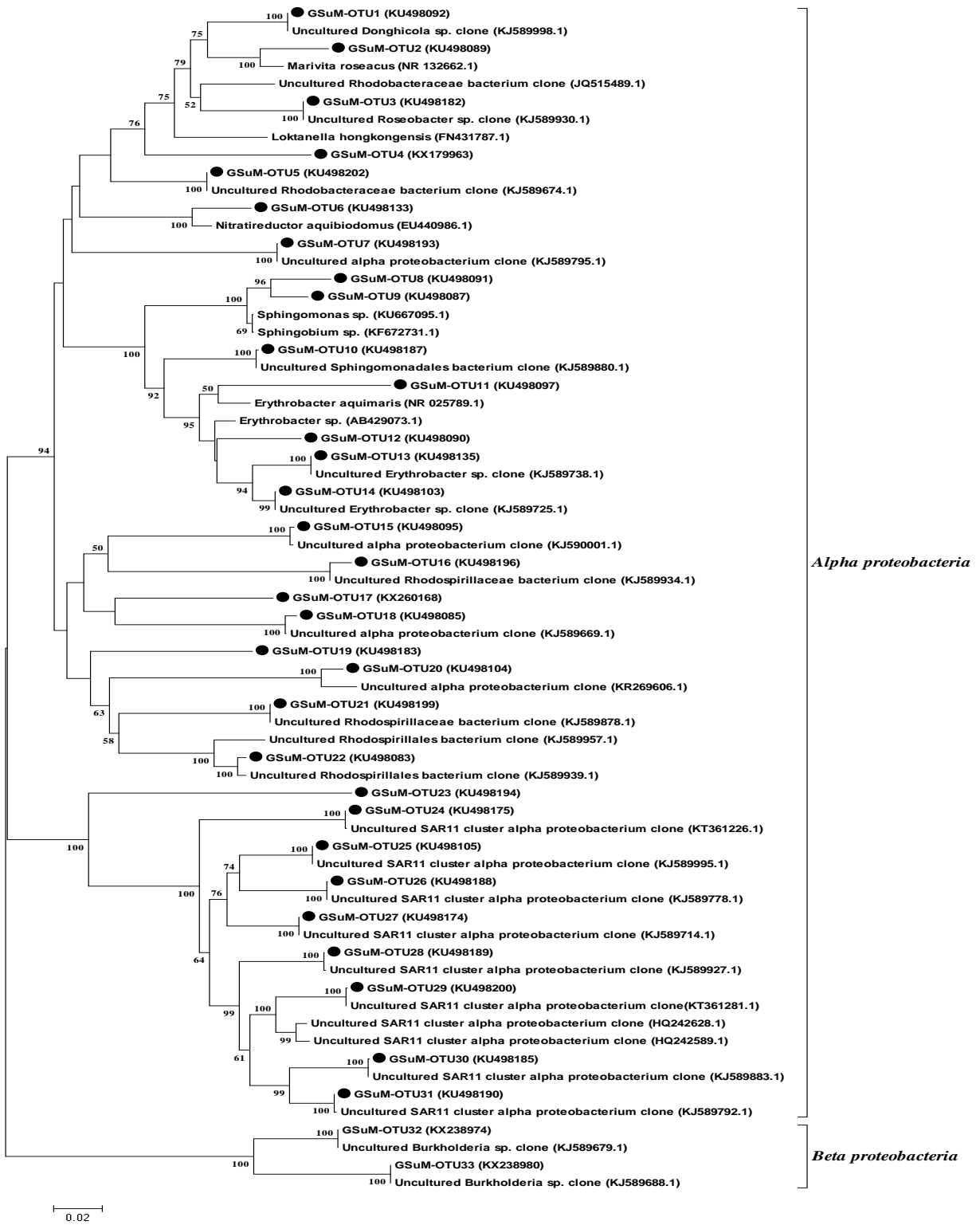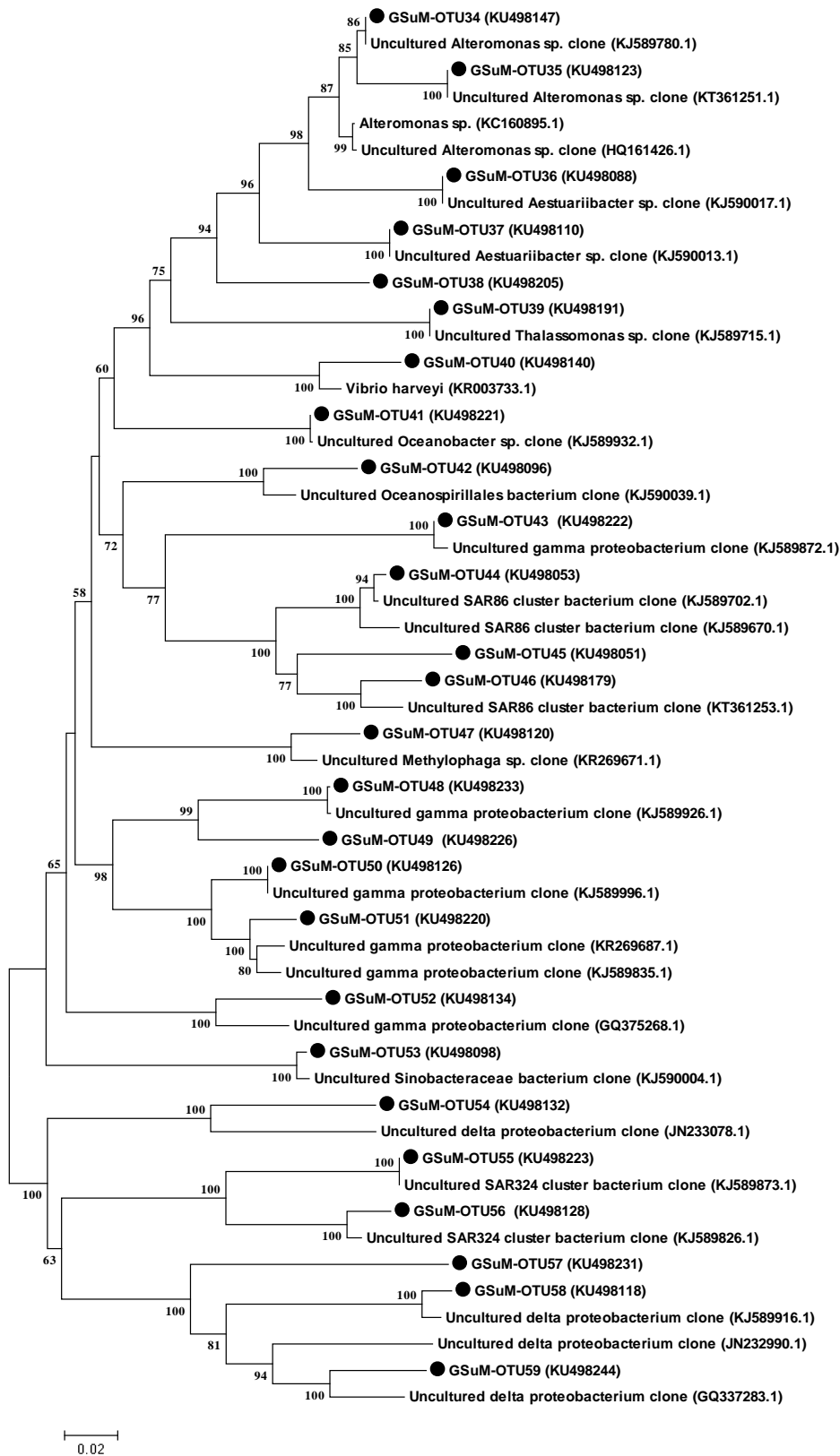
Fig 5a.3a

Fig 5a.3b

Fig 5a.3c

Figure 5a.3: Neighbor joining tree of 16S rRNA gene sequences obtained from the water samples collected off Goa during SuM: Summer Monsoon. Sequences are aligned with the best matches available in the Genbank. Tree topology was inferred by using 1000 bootstrap iterations. Bootstrap values above 50% at each node. Scale bar represents the nucleotide substitution percentage.

    a) Alphaproteobacteria and Betaproteobacteria

    b) Gammaproteobacteria and Deltaproteobacteria

    c) Acidobacteria, Marinimicrobia, Actinobacteria Verrucomicrobia and Cyanobacteria

5a.3.2.3 FIM: 103 OTUs were formed from the 290 clones analyzed during FIM. (30 OTUs; 74 clones) belonged to *Alphaproteobacteria* affiliating to *Roseibacterium elongatum, Loktanella hongkongensis, Rhodobacteraceae, Rhodospirillaceae Donghicola, Nitratireductor, Maricaulis, Erythrobacter, Sphingomonas* sp., SAR11 cluster and uncultured *Alphaproteobacterium.* (28 OTUs; 107 sequences) belonged to *Gammaproteobacteria* with clones affiliating to *Idiomarina baltica, Oleibacter marinus, Pseudomonas aeruginosa, Oceanospirillales, Marinobacter, Halomonas, Alteromonas, Pseudolteromonas, Methylophaga, Vibrio* sp., and uncultured *Gammaproteobacteria. Deltaproteobacteria* (10 OTUs; 22 sequences) belonged to SAR324 cluster and uncultured *Deltaproteobacterium, Marinimicrobia* (5 OTUs; 6 sequences), affiliating to *Deferribacteres* sp. and uncultured *Marinimicrobia* sp., *Firmicutes* (4 OTUs; 4 sequences) belonged to *Bacillus* sp., *Betaproteobacteria* (1 OTU; 3 sequences) belonged to *Burkolderia ambifaria, Acidobacteria* (6 OTUs; 9 sequences)*, Cyanobacteria* (6 OTUs; 36 sequences), *Bacteroidetes* (2 OTUs; 3 sequences), *Chloroflexi* and *Omnitrophica bacterium* (1 OTU; 4 sequences), *Planctomycetes* (1 OTU; 4 sequences), *Actinobacteria* (5 OTUs; 15 sequences) and *Verrucomicrobia* (3 OTUs; 3 sequences) (Fig 5a.4a, 5a.4b, 5a.4c, 5a.4d and 5a.4e).

Fig 5a.4 a

Fig 5a.4 a — Phylogenetic tree of Gamma proteobacteria

- 94 ◼ GFIM-OTU31 (KT361366)
- Alteromonas macleodii strain (KM037750.1)
- Uncultured Alteromonas sp. clone (HQ161365.1)
- Uncultured Alteromonas sp. clone (HQ161374.1)
- 91 ◼ GFIM-OTU32 (KP076546)
- 80 ◼ GFIM-OTU33 (KT361348)
- ◼ GFIM-OTU34 (KT361352)
- Alteromonas macleodii strain(KU052036.1)
- 50 51 Uncultured Alteromonas sp. clone (HQ161367.1)
- 77 ◼ GFIM-OTU35 (KP076462)
- ◼ GFIM-OTU36 (KP076439)
- 100 ◼ GFIM-OTU37 (KT361340)
- 100 Uncultured Alteromonas sp. clone (KJ589656.1)
- Pseudoalteromonas sp. (GQ495024.1)
- 70 Alteromonas sp. (KT215604.1)
- 67 ◼ GFIM-OTU38 (KP076460)
- 100 Uncultured Alteromonadaceae bacterium clone (KJ590041.1)
- 68 ◼ GFIM-OTU39 (KP076476)
- 92 Methylophaga sp. (KF371656.1)
- Uncultured Methylophaga sp. clone (JN860321.1)
- 100 ◼ GFIM-OTU40 (KT361369)
- 92 ◼ GFIM-OTU41 (KT361343)
- 100 Uncultured Methylophaga sp. clone (KT361230.1)
- 100 ◼ GFIM-OTU42 (KT361388)
- Idiomarina baltica (KM407692.1)
- ◼ GFIM-OTU43 (KT361378)
- 87 ◼ GFIM-OTU44 (KT361333)
- 64 Vibrio brasiliensis strain (KT428055.1)
- 100 Vibrio caribbeanicus strain (NR 108732.1)
- 92 ◼ GFIM-OTU45 (KT361364)
- 60 Vibrio chagasii (LN832959.1)
- ◼ GFIM-OTU46 (KT361326)
- 83 Uncultured Vibrio sp. clone (KF758747.1)
- ◼ GFIM-OTU47 (KP076551)
- 99 Oleibacter marinus strain (NR 114287.1)
- ◼ GFIM-OTU48 (KP076502)
- ◼ GFIM-OTU49 (KP076451)
- 81 Uncultured gamma proteobacterium clone (FM242233.1)
- ◼ GFIM-OTU50 (KP076509)
- 100 ◼ GFIM-OTU51 (KP076447)
- 98 Uncultured gamma proteobacterium clone (GQ337220.1)
- 56 Uncultured gamma proteobacterium clone (KJ589996.1)
- 99 ◼ GFIM-OTU52 (KP076531)
- 100 Uncultured gamma proteobacterium clone (KJ589830.1)
- 77 ◼ GFIM-OTU53 (KP076554)
- 98 Uncultured gamma proteobacterium clone (KJ589839.1)
- 100 ◼ GFIM-OTU54 (KP076489)
- Uncultured gamma proteobacterium clone (EU328045.1)
- ◼ GFIM-OTU55 (KP076539)
- 100 Pseudomonas aeruginosa strain (KU508628.1)
- 100 ◼ GFIM-OTU56 (KP076531)
- Halomonas sp. (FJ785508.1)
- 100 ◼ GFIM-OTU57 (KP076497)
- Uncultured Oceanospirillales bacterium clone (KJ590039.1)
- 76 ◼ GFIM-OTU58 (KP076465)
- 100 Marinobacter sp. (AB985581.1)
- Uncultured Verrucomicrobia bacterium clone (KJ589906.1)

Gamma proteobacteria

0.02

Fig 5a.4 b
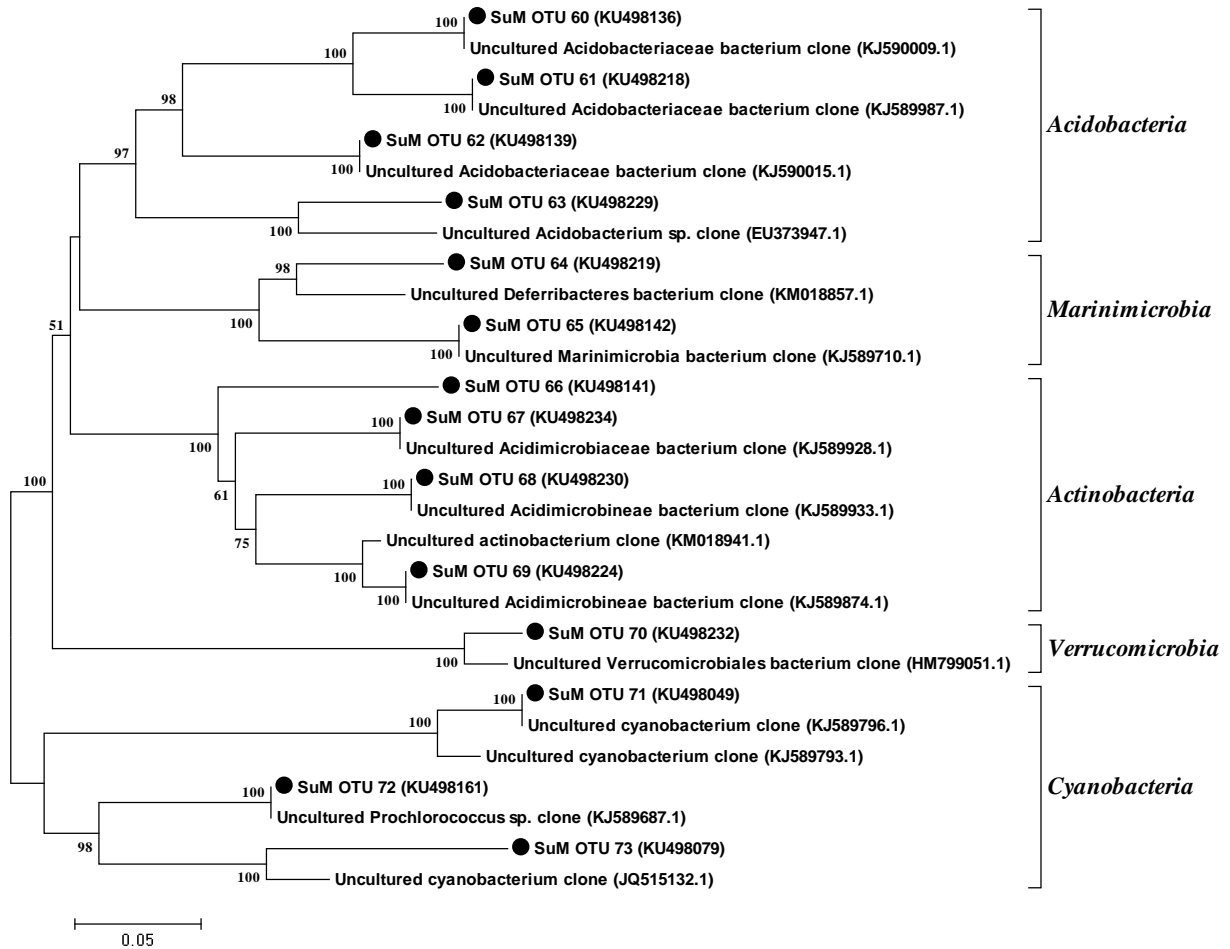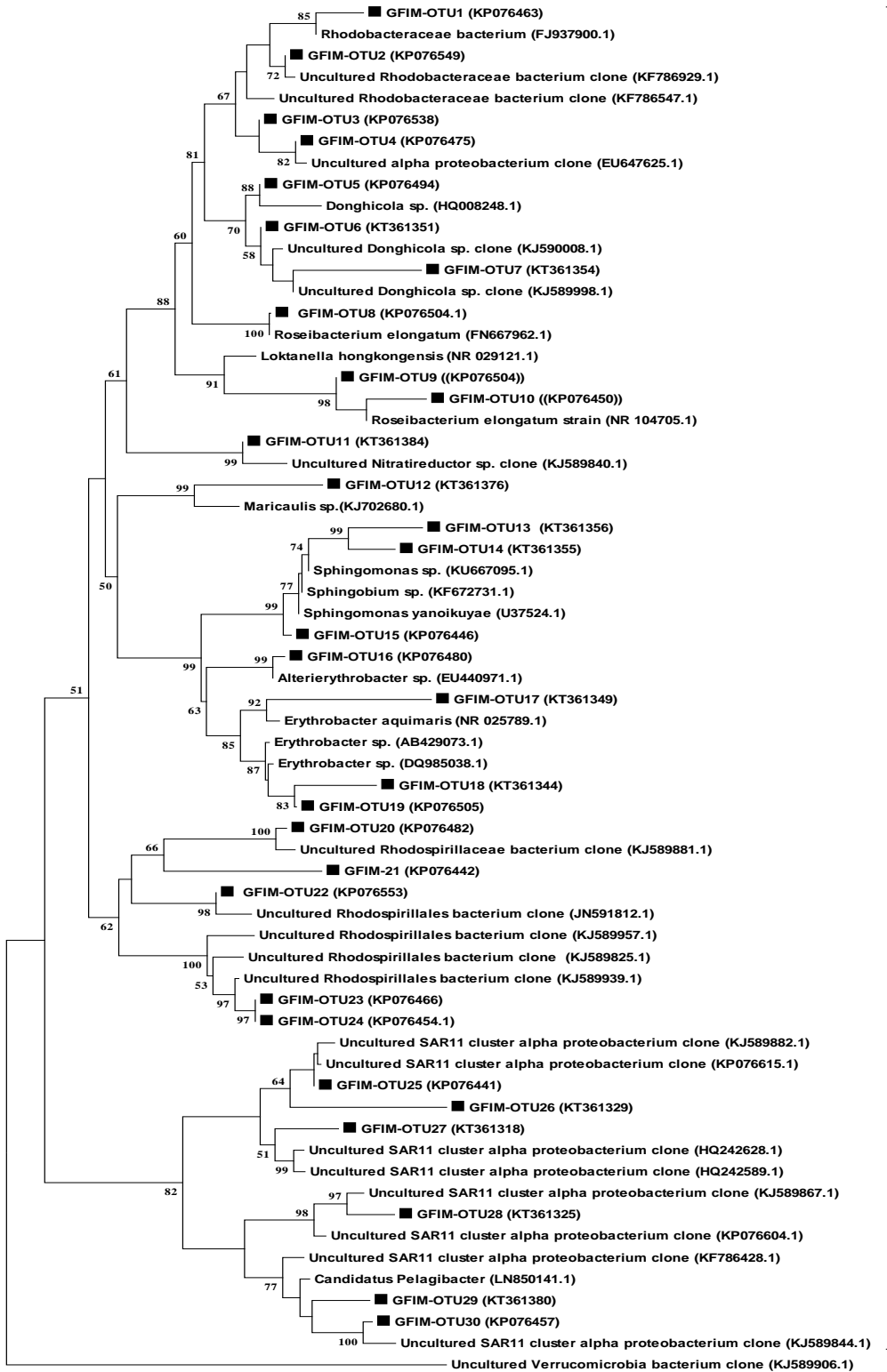
109

Fig 5a.4 c

Fig 5a.4 d

Fig 5a.4 e

Figure 5a.4: Neighbor joining tree of 16S rRNA gene sequences obtained from the water samples collected off Goa during FIM: Fall Inter-Monsoon. Sequences are aligned with the best matches available in the Genbank. Tree topology was inferred by using 1000 bootstrap iterations. Bootstrap values above 50% at each node. Scale bar represents the nucleotide substitution percentage.

   a) Alphaproteobacteria

   b) Gammaproteobacteria

   c) Deltaproteobacteria and Betaproteobacteria

   d) Acidobacteria, Omnitrophica bacterium,Planctomycetes, Cyanobacteria, Chloroflexi and Bacteroidetes

111

e) Marinimicrobia, Firmicutes, Actinobacteria and Verrucomicrobia

5a.3.3 Statistical analysis of 16S rRNA clone libraries

The Chao1 estimator of species diversity, Shannon diversity index, Simpson diversity and Good's coverage for each sampling site were calculated (Table 5a.1). The Shannon and Simpson diversity indices were the highest during FIM (4.2 and 0.01, respectively) followed by SIM (3.8 and 0.06, respectively) and the lowest during SuM (3.22 and 0.10, respectively). The estimated coverage values ranged from 84% to 90%. The rarefaction analysis was done for the comparing the sampling effort and phylotype obtained (Fig 5a.5) indicated that more ribogroups were found during FIM and the least during SuM at equal sampling effort.

Table 5a.1: Distribution of ribogroups, diversity indices and coverage of OTUs during different seasons from coastal waters of the central Arabian Sea off Goa

Diagnostic	SIM	SuM	FIM
No. of sequences	290	290	290
No. of OTUs	86	73	103
Shannon's index	3.84	3.22	4.2
Simpson's index	0.06	0.10	0.01
Chao1	97.68	94.66	135.62
Good's coverage (%)	90	88	84
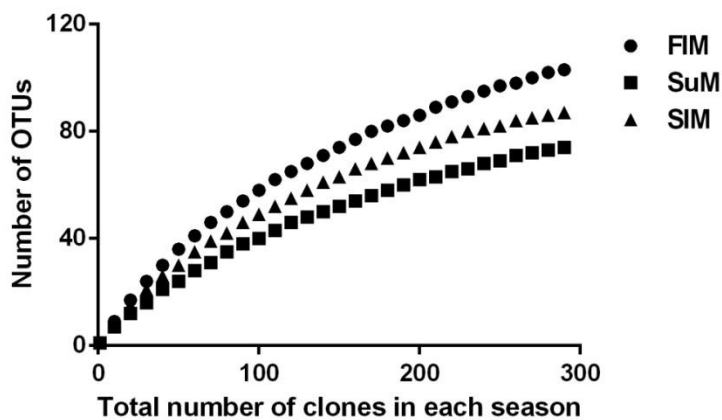
Fig. 5a.5: Rarefaction curves of operational taxonomic units (OTU) of 16SrRNA gene sequences obtained in each season.

METAGENOMIC DNA ANALYSIS BASED BACTERIAL COMMUNITY STRUCTURE OFF MANGALORE

5b.1 <u>Introduction</u>

Coastal waters harbor a tremendous diversity of microbes able to transform C-, N-,P- and S-containing compounds. The metabolism of marine microbes plays significant roles in the functioning of the ecosystem and maintains the biogeochemical cycles on the Earth. Understanding the relation between the distribution of microbial diversity and ecosystem functioning is necessary to predict the responses of the ecosystem to the changing environment.

5b.2 <u>Materials and methods</u>

5b.2.1 Sample Collection

Details on the location of the study area and its details are specified in Chapter 3, section 3.2.2. For phylogenetic analyses, sea water collected from M8 (Off Mangalore) during all three seasons was used.

5b.2.2 Extraction of Metagenomic DNA

The methodology for is described in Section 5a.2.2

5b.2.3 PCR amplification of 16S rRNA gene from DNA extracts

Details of PCR amplification is described in section 3.2.7

5b.2.4 Clone library construction; DNA sequencing, phylogenetic tree construction and statistical analysis

The methodology for is already described in Section 5a.2.4 and 5a.2.5

5b.2.5 Nucleotide sequence accession numbers

Sequences were submitted to GenBank and accession numbers assigned are KP076560 to KP076645, KT361396 to KT361409, KX987400 to KX987499 and KY561386- KY561585.

5b.3 <u>Results</u>

5b.3.1 Bacterial community structure Off Mangalore

A total of 300 clones obtained from each season were subjected to 16S rRNA gene sequencing. The sequences could be categorized into *Gammaproteobacteria*, *Alphaproteobacteria*, *Deltaproteobacteria*, *Betaproteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Marinimicrobia*, *Verrucomicrobia* and *Planctomycetes*.

5b.3.1.1 Seasonal variations in bacterial community

Seasonal comparisons discerned the fact that bacteria were diverse during FIM followed by SuM and less diverse during SIM. *Gammaproteobacteria, Alphaproteobacteria* and *Cyanobacteria* were found during all three seasons. Clones of *Marinimicrobia* and *Bacteroidetes* were observed during SIM and FIM, while *Acidobacteria, Verrucomicrobia* and *Actinobacteria* were found mainly during SuM and FIM. *Betaproteobacteria* and *Deltaproteobacteria* were found exclusively during FIM.

5b.3.1.2 Major Phylogenetic groups during SIM

Five different Phylogenetic groups were found during SIM: *Gammaproteobacteria*, *Alphaproteobacteria*, *Bacteroidetes*, *Cyanobacteria* and *Marinimicrobia*. *Gammaproteobacteria* was the major group observed during SIM found throughout the water column, particularly at the surface. *Cyanobacteria* was evident at the surface along with *Gammaproteobacteria*. It was observed that bacterial groups were found to be diverse towards the bottom. *Alphaproteobacteria*, *Marinimicrobia* and *Bacteroidetes* were mainly observed at the near bottom depth (Fig. 5b.1a).

5b.3.1.3 Major Phylogenetic groups during SuM

During SuM, six different Phylogenetic groups were identified: *Gammaproteobacteria*, *Alphaproteobacteria*, *Actinobacteria, Acidobacteria, Cyanobacteria* and *Verrucomicrobia*. *Gammaproteobacteria* and *Alphaproteobacteria* were the dominant groups. *Alphaproteobacteria* was observed throughout the water column. *Gammaproteobacteria, Actinobacteria* and *Acidobacteria* were found in the subsurface waters. Minor group *Verrucomicrobia* was observed only at the bottom depth (Fig. 5b.1a).

5b.3.1.4 Major Phylogenetic groups during SIM

In total, Eleven bacterial phyla were evident during FIM with *Proteobacteria* being the major prominent group, followed by *Cyanobacteria* (Fig. 5b.1a). Phyla of *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Marinimicrobia*, *Verrucomicrobia* and *Planctomycetes* were also detected, but with much less abundance. *Bacteroidetes* and *Cyanobacteria* were particularly found at the surface. *Alphaproteobacteria* and *Gammaproteobacteria* were present at all the depths. Moving towards the bottom layers *Deltaproteobacteria* and *Gammaproteobacteria* intensified. The relative abundance of *Alphaproteobacteria* decreased at the bottom layer with the increase of relative abundance of Gammaproteobacteria (Fig. 5b.1a).
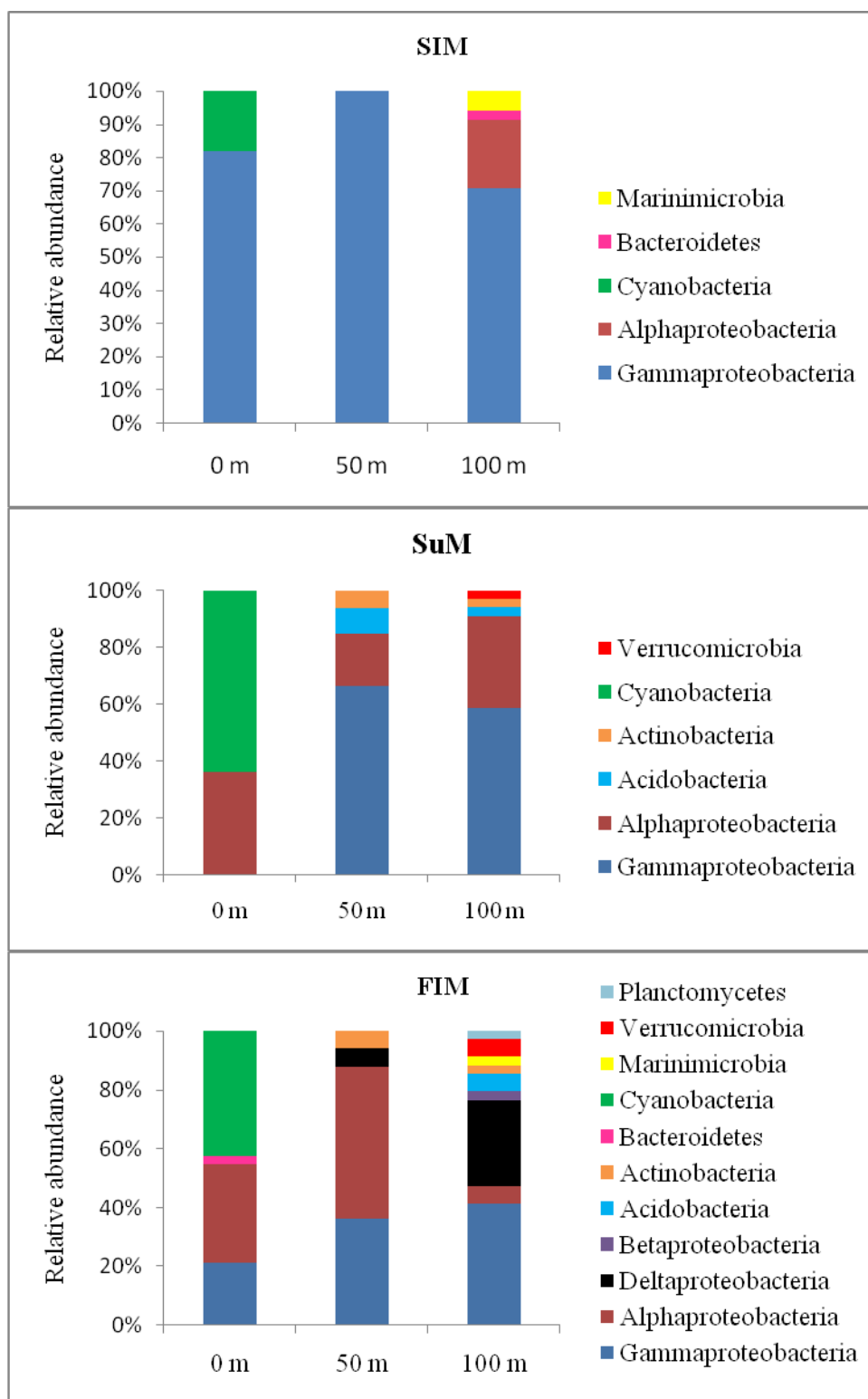
Fig.5b.1a: Depth profile of bacterial community structure Off Mangalore

5b.3.2 Phylogenetic analysis of bacterial 16SrRNA genes across all three seasons

Relationships between the dominant OTUs in each season and their closest neighbors were shown by Phylogenetic analyses, after grouping the sequences with MOTHUR.

5b.3.2.1 SIM: Phylogenetic analysis showed that 31 OTUs (or phylogroups) were obtained in all. A total of 17 OTUs; 84 clones represented *Gammaproteobacteria* with clones affiliating to *Pseudomonas aeruginosa, Photobacterium, Alteromonas, Pseudoalteromonas, Colwellia, Oceanobacter, Halomonas* sp. respectively and uncultured *gamma proteobacterium. Alphaproteobacteria* (6 OTUs; 7 sequences) affiliated to *Rhodospirillaceae,* SAR11 cluster, *Rhodobacteraceae* and uncultured *alpha proteobacterium, Cyanobacteria* (5 OTUs; 6 sequences), *Marinimicrobia* (2 OTUs) and one OTU belonged to *Bacteroidetes* (Fig. 5b.2a and 5b.2b).

Fig. 5b.2a

Fig. 5b.2b

Figure 5b.2: Neighbor joining phylogenetic tree inferred from 16S rRNA gene sequences obtained off Mangalore during SIM; Spring Inter-Monsoon. Values at nodes are the percent occurrence of the sequences in the same cluster (Bootstrap values <50 are not shown). Scale bar represents the nucleotide substitution percentage.

    a) Gammaproteobacteria

    b) Cyanobacteria, Marinimicrobia, Alphaproteobacteria and Bacteroidetes

5b.3.2.2 SuM: A total of 37 OTUs were obtained, Phylogenetic analyses showed that 15 OTUs out of 42 clones belonged to *Gammaproteobacteria* with the dominant OTUs belonging to *Alteromonas, Thalassomonas, Methylophaga, Colwellia, Pseudomonas* sp.

respectively and uncultured *gamma proteobacterium*. 16 OTUs out of 29 sequences belonged to *Alphaproteobacteria* with sequences affiliating to *Rhodobacteraceae, Rhodospirillales,* SAR11 cluster, *Erythrobacter, Sphingobium*, *Donghicola Nitratireductor* sp. respectively and *uncultured alpha proteobacterium. Cyanobacteria* (2 OTUs; 21 sequences), *Actinobacteria* (2 OTUs; 3 sequences), *Acidobacteria* (1 OTU; 4 sequences) and one OTU belonged to *Verrucomicrobia* sp. (Fig. 5b.3a and 5b.3b).

Fig. 5b.3a

Fig. 5b.3b

Figure 5b.3: Neighbor joining phylogenetic tree inferred from 16S rRNA gene sequences obtained off Mangalore during SuM; Summer Monsoon. Values at nodes are the percent

occurrence of the sequences in the same cluster (Bootstrap values <50 are not shown). Scale bar represents the nucleotide substitution percentage.

a) Gammaproteobacteria and Actinobacteria
b) Alphaproteobacteria, Acidobacteria, Verrucomicrobia and Cyanobacteria

5b.3.2.3 FIM: During FIM 49 OTUs were obtained overall. 12 OTUs; 33 clones belonged to *Gammaproteobacteria* with clones affiliating to *Alteromonas, Vibrio, Halomonas, Colwellia* and *Oceanobacter* sp. respectively and uncultured *gamma proteobacterium.* 16 OTUs out of 30 clones were divided among *Alphaproteobacteria* with clones belonging to SAR11 cluster, *Rhodospirillaceae, Erythrobacter, Sphingomonas, Mesorhizobium, Loktanella, Nautella* and *Roseobacter* sp. respectively and uncultured *alpha proteobacterium, Cyanobacteria* (6 OTUs; 16 sequences), *Deltaproteobacteria* (6 OTUs; 10 sequences), *Actinobacteria* (3 OTUs; 3 sequences), *Acidobacteria* and *Verrucomicrobia* (1 OTU ; 2 sequences each) and one OTU each belonging to *Betaproteobacteria* affiliating to *Burkholderia ambifaria, Planctomycetes, Bacteroidetes* and *Marinimicrobia* (Fig 5b.4a and 5b.4b).

62 ■ MFIM-OTU 1 (KT361402)

Candidatus Pelagibacter sp.(LN850154.1)

■ MFIM-OTU 2 (KP076571)

■ MFIM-OTU 3 (KP076574)

70 Uncultured SAR11 cluster alpha proteobacterium clone (KJ589927.1)

65

87 ■ MFIM-OTU 4 (KT361400)

Uncultured SAR11 cluster alpha proteobacterium clone (HQ242628.1)

Uncultured SAR11 cluster alpha proteobacterium clone (HQ242374.1)

69

87 ■ MFIM-OTU 5 (KP076632)

89 Uncultured SAR11 cluster alpha proteobacterium clone (KJ589980.1)

75 ■ MFIM-OTU 6 (KP076604)

Uncultured SAR11 cluster alpha proteobacterium clone (KJ589867.1)

52 Uncultured SAR11 cluster alpha proteobacterium clone (DQ810524.1)

86

80 ■ MFIM-OTU 7 (KP076595)

84 Uncultured SAR11 cluster alpha proteobacterium clone (KJ589714.1)

■ MFIM-OTU 8 (KP076603)

95 ■ MFIM-OTU 9 (KP076591)

Uncultured alpha proteobacterium clone (KJ589859.1)

■ MFIM-OTU 10 (KP076638)

98 Uncultured Rhodospirillaceae bacterium clone (KJ589909.1)

98 ■ MFIM-OTU 11 (KP076564)

Erythrobacter sp. (AB429073.1)

99

■ MFIM-OTU 12 (KP076614)

100 Sphingomonas sp. (KU667095.1)

99 ■ MFIM-OTU 13 (KP076594)

Mesorhizobium sp. (EU937747.1)

99 ■ MFIM-OTU 14 (KP076567)

Loktanella hongkongensis strain (NR 029121.1)

56

50

91 ■ MFIM-OTU 15 (KP076600)

Uncultured Nautella sp. (JN087482.1)

94

75 ■ MFIM-OTU 16 (KP076611)

100 Uncultured Roseobacter sp. clone (KJ589930.1)

Alpha proteobacteria

94 ■ MFIM-OTU 17 (KP076641)

96 Uncultured gamma proteobacterium clone (GQ337142.1)

■ MFIM-OTU 18 (KP076578)

98 Uncultured gamma proteobacterium clone (KJ589841.1)

97

90 ■ MFIM-OTU 19 (KP076592)

Uncultured gamma proteobacterium clone (KJ589839.1)

93 ■ MFIM-OTU 20 (KP076601)

89 Uncultured gamma proteobacterium clone (KJ589829.1)

99 ■ MFIM-OTU 21 (KP076609)

98 Uncultured Oceanobacter sp. clone (KJ589932.1)

■ MFIM-OTU 22 (KP076596)

91 Uncultured Oceanobacter sp. clone (KJ589922.1)

85

■ MFIM-OTU 23 (KP076563)

99 Halomonas sp. (FJ785508.1)

93 ■ MFIM-OTU 24 (KT361408)

53 Vibrio sp. (FJ457591.1)

96

88 Uncultured Vibrio sp. clone (KM873137.1)

■ MFIM-OTU 25 (KT361406)

87

98 ■ MFIM-OTU 26 (KP076593)

Uncultured Colwellia sp. clone (KJ589973.1)

90

65 ■ MFIM-OTU 27 (KP076570)

Alteromonas macleodii strain (KU052036.1)

96

82 ■ MFIM-OTU 28 (KP076612)

57 Uncultured Alteromonas sp. clone (JN886917.1)
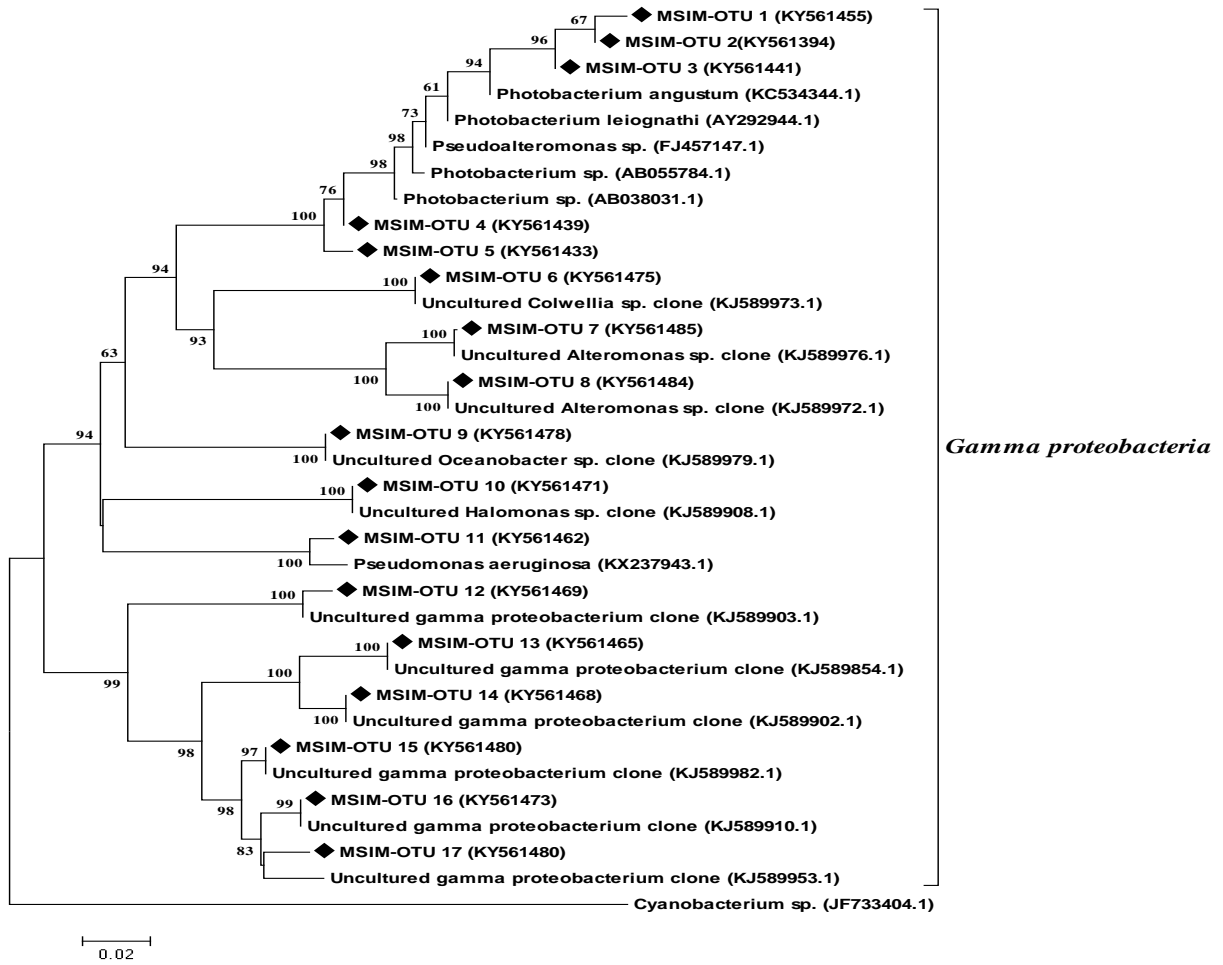
Gamma proteobacteria
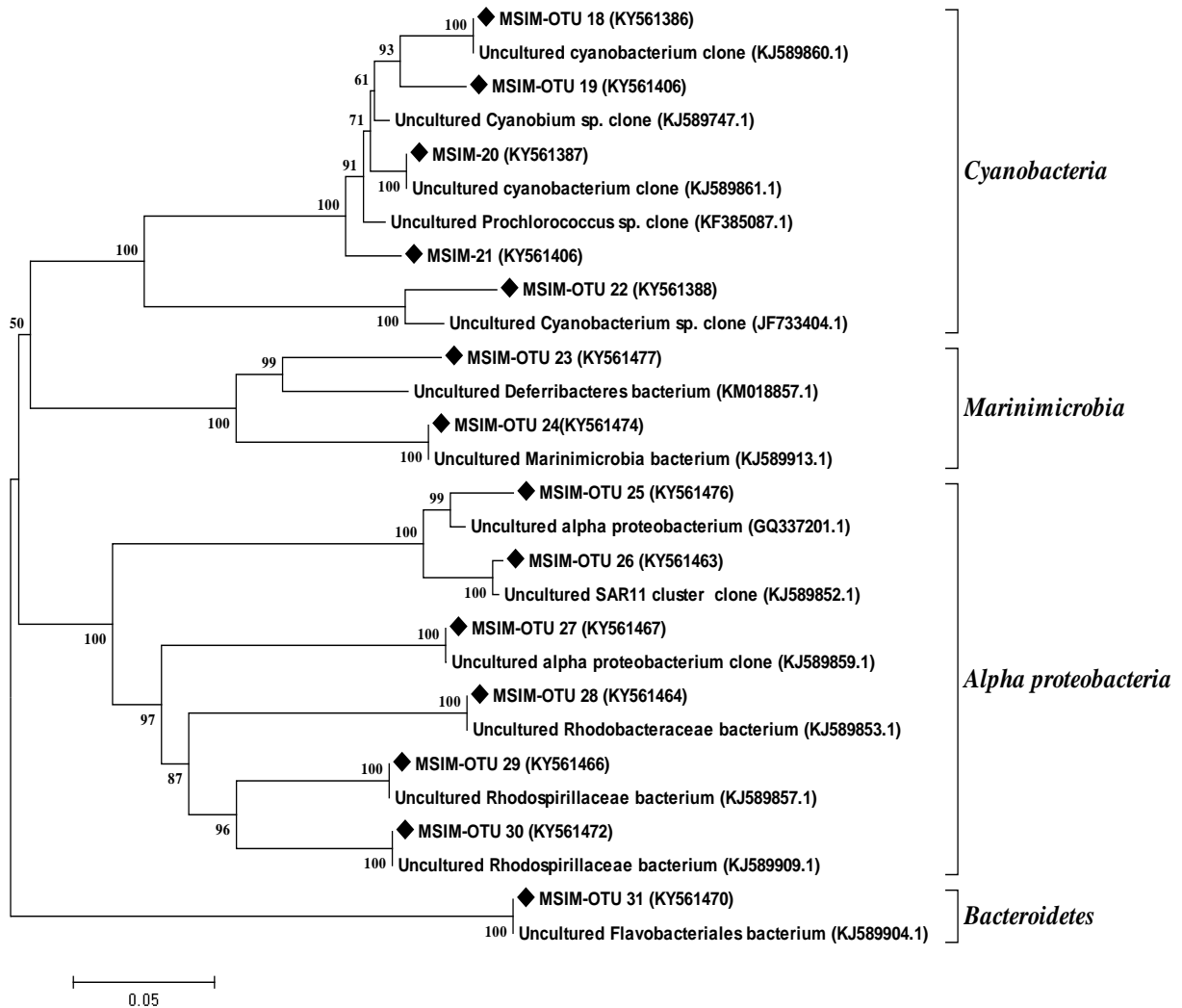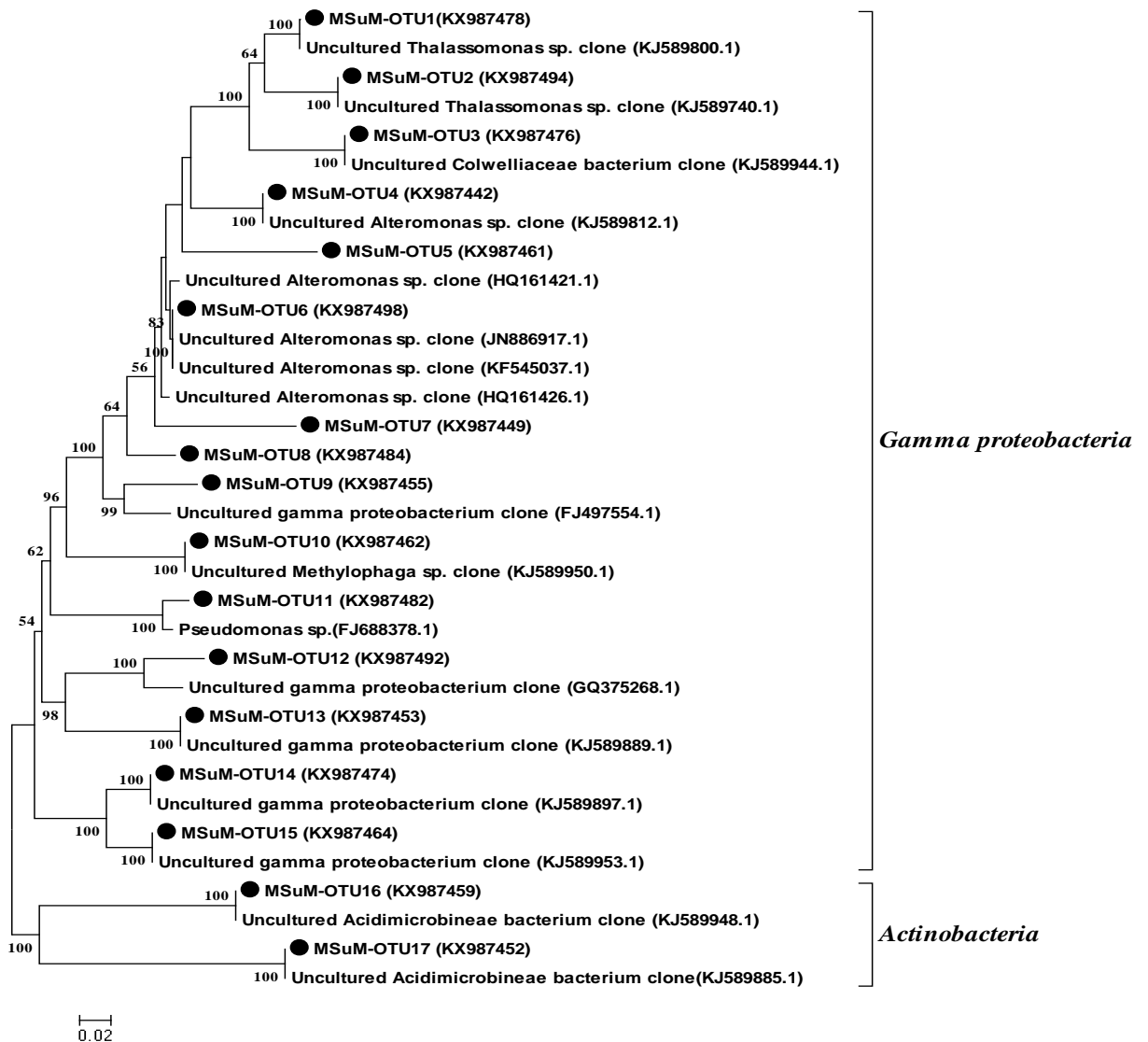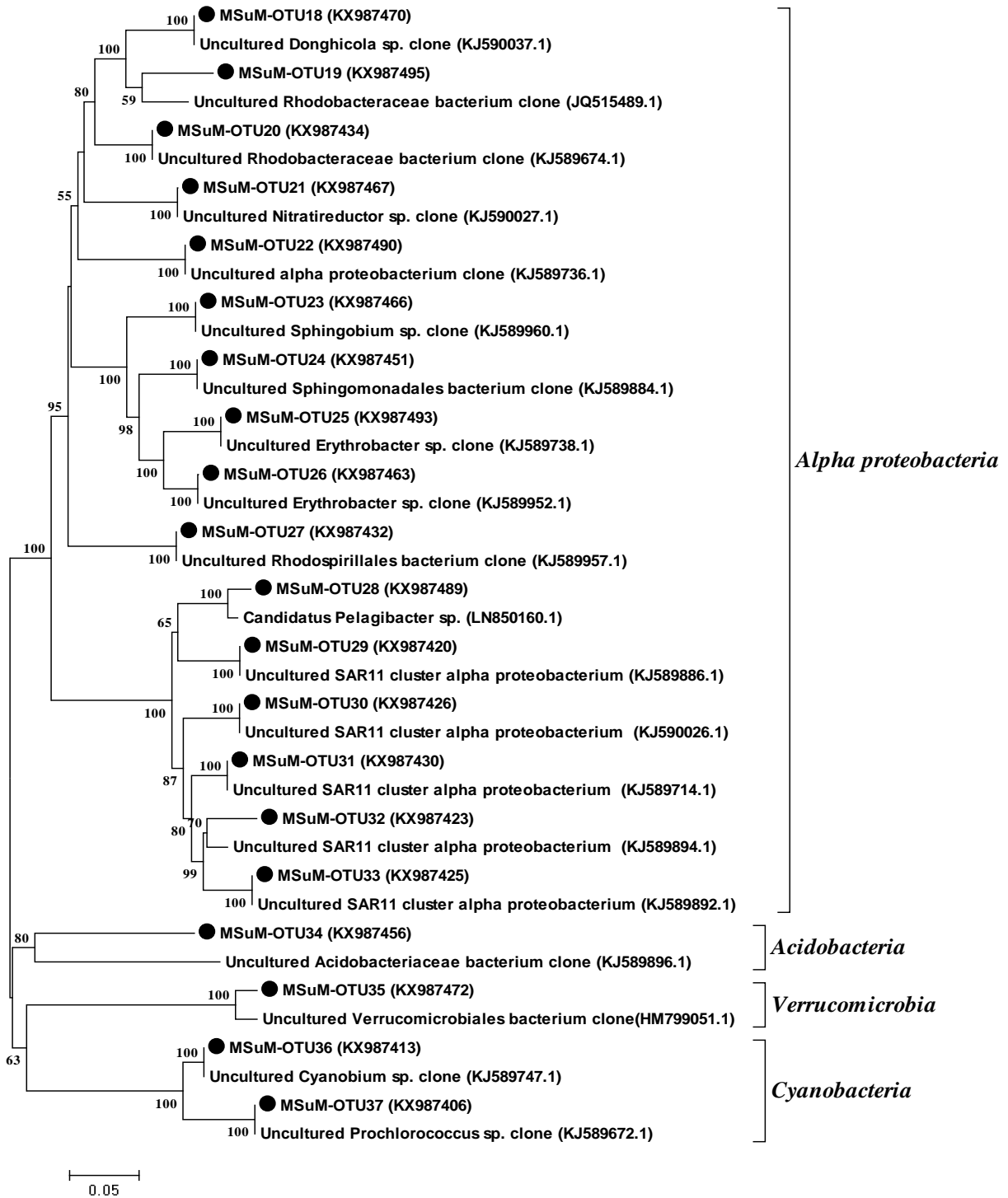
0.02

Fig. 5b.4a

122

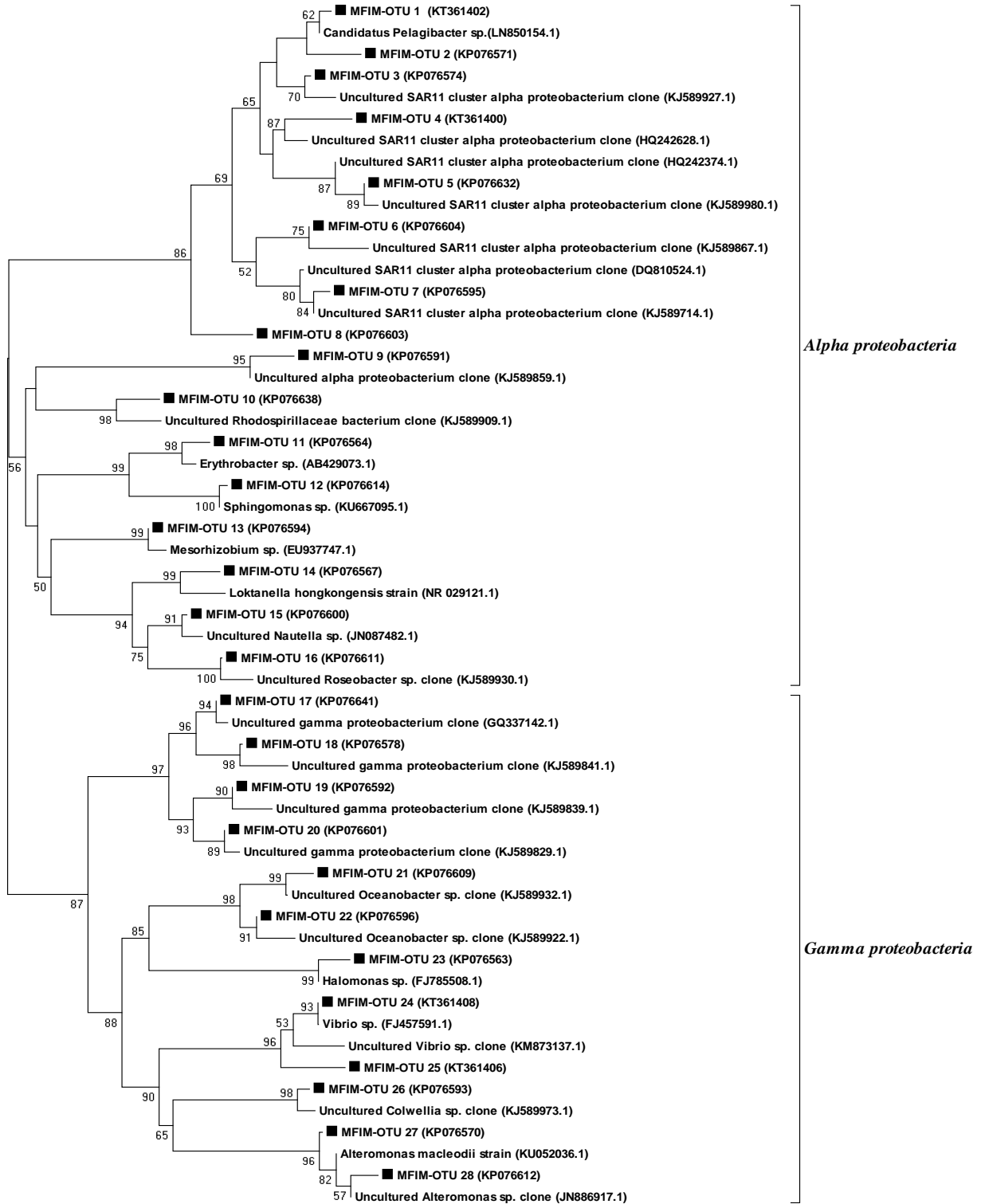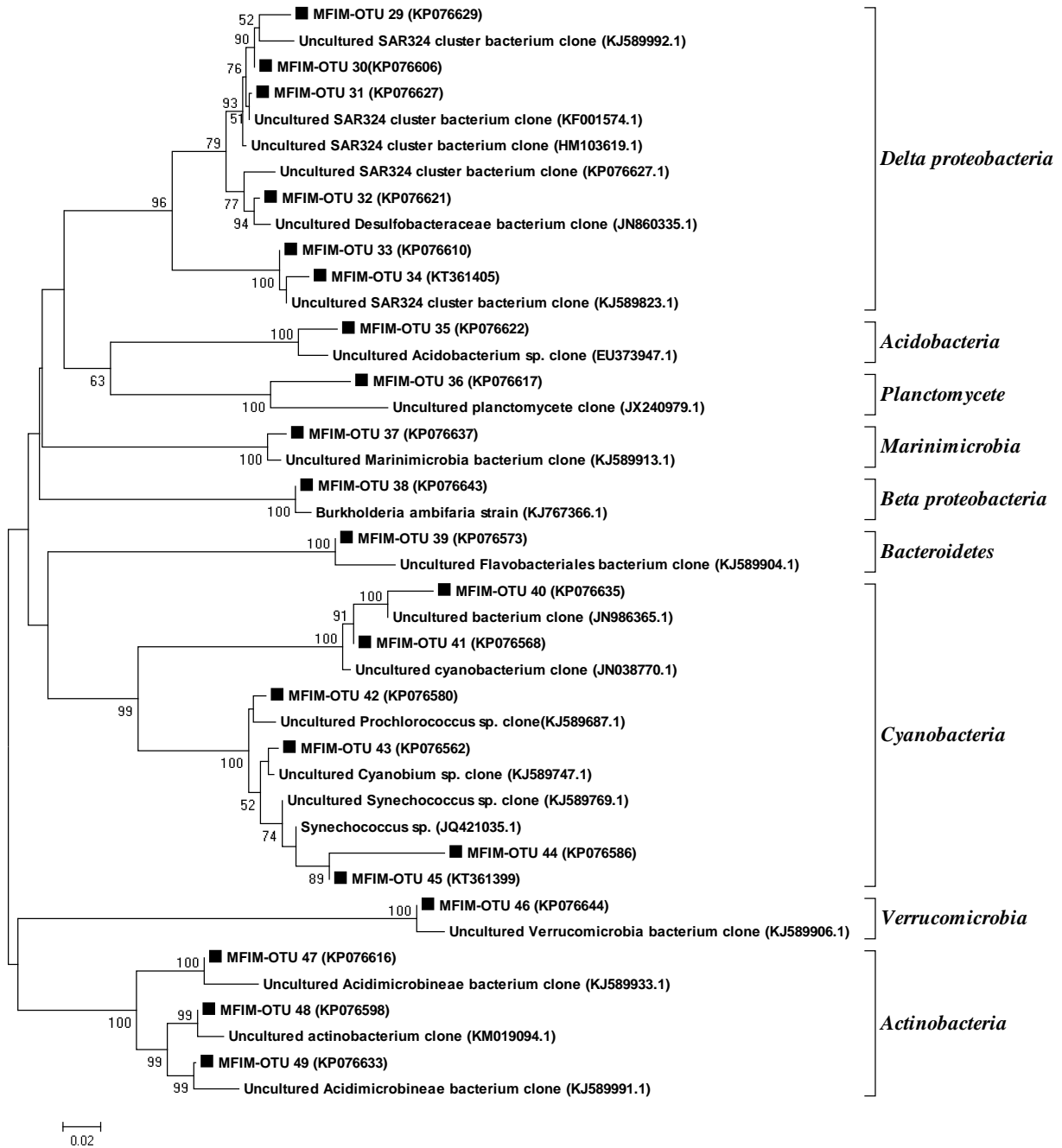**Fig. 5b.4b**

Figure 5b.4: Neighbor joining phylogenetic tree inferred from 16S rRNA gene sequences obtained off Mangalore during FIM; Fall Inter-monsoon. Values at nodes are the percent occurrence of the sequences in the same cluster (Bootstrap values <50 are not shown). Scale bar represents the nucleotide substitution percentage.

a) Alphaproteobacteria and Gammaproteobacteria

b) Deltaproteobacteria, Acidobacteria, Planctomycetes, Marinimicrobia, Betaproteobacteria, Bacteroidetes, Cyanobacteria, Verrucomicrobia and Actinobacteria

5b.3.3 Statistical analysis of 16S rRNA clone libraries

Both Shannon and Simpson diversity indices were the highest during FIM (3.59 and 0.02, respectively) followed by SuM (2.84 and 0.11, respectively) and the lowest during SIM (2.35 and 0.19, respectively). The estimated coverage values ranged from 72% to 76% (Table 5b.). The rarefaction analysis indicated that more ribogroups were found during FIM and the least during SIM at equal sampling effort (Fig 5b.5).

Table 5b.1: Distribution of ribogroups, diversity indices and coverage of OTUs during different seasons from coastal waters of the eastern Arabian Sea off Mangalore

Diagnostic	SIM	SuM	FIM
No. of sequences	100	100	100
No. of OTUs	31	37	49
Shannon's index	2.35	2.84	3.59
Simpson's index	0.19	0.11	0.02
Chao1	128.5	76.42	74.07
Good's coverage (%)	72	76	73

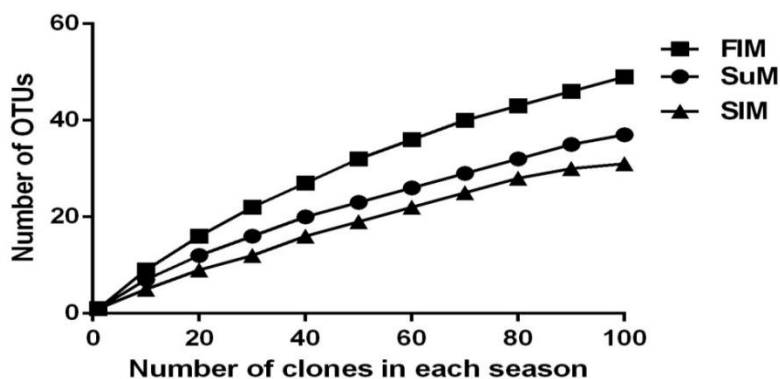**Fig. 5b.5:** Rarefaction curves of operational taxonomic units (OTU) of 16SrRNA gene sequences obtained in each season.

METAGENOMIC DNA ANALYSIS BASED BACTERIAL COMMUNITY STRUCTURE OFF KOCHI

5c.1 <u>Introduction</u>

The west coast of India known for intense productivity in response to upwelling harbors microbes that cannot be cultivated. Such microbes are of great significance to the environment and respond rather shortly to changing environmental conditions. Culture-independent studies based on 16S rRNA gene sequencing is useful to discern the vast and uncharacterized bacterial diversity (Alonso-Gutiérrez et al. 2009; Cury et al. 2011). The coastal ecosystem experiencing seasonal hypoxia are influenced by seasonal upwelling and nutrients brought in from the adjacent terrestrial ecosystems.

5c.2 <u>Materials and methods</u>

5c.2.1 Sample Collection

Details of location of the study area and its details are specified in Chapter 3, section 3.2.2. For phylogenetic analyses, sea water collected from K3 (Off Kochi) during all three seasons was used.

5c.2.2 Extraction of Metagenomic DNA

The methodology for is described in Section 5a.2.2

5c.2.3 PCR amplification of 16S rRNA gene from DNA extracts

Details of PCR amplification is described in section 3.2.7

5c.2.4 Clone library construction; DNA sequencing, phylogenetic tree construction and statistical analysis

The methodology for is already described in Section 5a.2.4 and 5a.2.5

5c.2.5 Nucleotide sequence accession numbers

Sequences were submitted to GenBank and accession numbers assigned are KY561486 to KY561585, KX987500 to KX987599 and KT361218 to KT361317.

5c.3 Results

5c.3.1 Bacterial community structure Off Kochi

After chimera removal a total of 300 clones obtained during SIM, SuM and FIM were subjected to 16S rRNA gene sequencing following which the sequences could be categorized into 13 types: *Gammaproteobacteria*, *Alphaproteobacteria*, *Deltaproteobacteria*, *Betaproteobacteria*, *Acidobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Cyanobacteria*, *Marinimicrobia*, *Verrucomicrobia*, *Planctomycetes* and *Chloroflexi*.

5c.3.1.1 Seasonal variations in bacterial community

Seasonal comparisons were useful to make out that the bacteria were diverse during FIM followed by SIM and less diverse during SuM. *Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria*, *Marinimicrobia* and *Cyanobacteria* were found during all three seasons. Clones of *Firmicutes, Actinobacteria*, *Acidobacteria* and *Verrucomicrobia* were observed during SIM and FIM, while, *Deltaproteobacteria* and *Chloroflexi* were found mainly during SuM and FIM. *Bacteroidetes* and *Planctomycetes* were found exclusively during FIM. *Gammaproteobacteria* was the major group observed in all three seasons.

5c.3.1.2 Major Phylogenetic groups during SIM

Nine distinct Phylogenetic groups were found during SIM: *Gammaproteobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Cyanobacteria*, *Firmicutes*, *Bacteroidetes*, *Marinimicrobia*, *Actinobacteria*, *Acidobacteria* and *Verrucomicrobia*. *Cyanobacteria* being the major group at the surface, along with *Betaproteobacteria* were seen at the surface. It was observed that bacterial groups were found to be diverse towards the bottom. *Firmicutes* showed an increasing trend moving towards the bottom. *Marinimicrobia, Acidobacteria* and

Actinobacteria were found at the subsurface waters and intensified at the bottom. *Alphaproteobacteria* was evident exclusively at the mid layer (Fig. 5c.1a).

5c.3.1.3 Major Phylogenetic groups during SuM

The Phylogenetic groups were found during SuM were *Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Cyanobacteria, Marinimicrobia* and *Chloroflexi. Cyanobacteria* was the exclusive group observed at the oxygenated surface layer. *Gammaproteobacteria* and *Alphaproteobacteria* were evident the subsurface waters. *Betaproteobacteria* and *Chloroflexi* were seen at the mid layer, *Deltaproteobacteria* and Marinimicrobia were found only at the bottom waters (Fig. 5c.1a).

5c.3.1.4 Major Phylogenetic groups during FIM

As compared to SIM and SuM, more bacterial group were found during FIM. 13 distinct Phylogenetic groups were found during FIM: *Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Cyanobacteria, Firmicutes, Bacteroidetes, Marinimicrobia, Planctomycetes, Chloroflexi, Actinobacteria, Acidobacteria* and *Verrucomicrobia.* The bacterial flora intensified at the subsurface layer as compared to the surface layers. *Gammaproteobacteria* and *Alphaproteobacteria* were found at all depths. *Deltaproteobacteria, Betaproteobacteria, Actinobacteria, Acidobacteria, Firmicutes, Marinimicrobia, Verrucomicrobia* and *Chloroflexi* were observed only at the bottom layer, except *Bacteroidetes* found at the mid layer (Fig. 5c.1a).
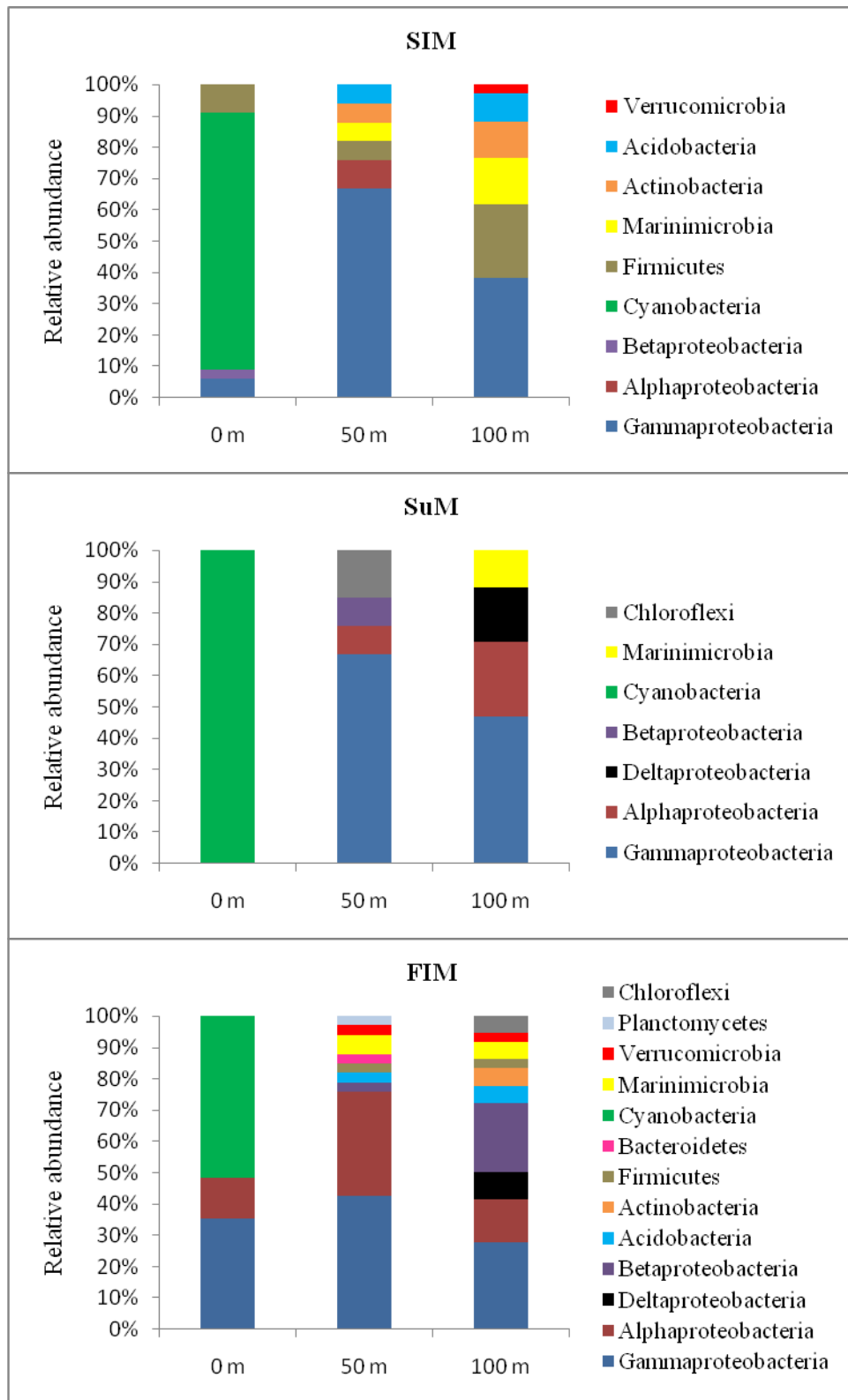
Fig.5c.1a: Bacterial community structure during different seasons Off Kochi

5c.3.2 Phylogenetic analysis of bacterial 16SrRNA genes across all three seasons

After grouping the dominant OTUs of each season in MOTHUR, the relationships between the obtained OTUs and their closest neighbors were deduced by constructing Phylogenetic trees.

SIM: Phylogenetic analyses showed that 37 OTUs were divided among *Gammaproteobacteria* (12 OTUs; 37 clones) with clones affiliated to *Marinobacter hydrocarbonoclasticus*, *Alteromonas*, *Pseudoalteromonas, Colwellia, Oceanobacter, Halomonas* sp. respectively and uncultured *gamma proteobacterium. Alphaproteobacteria* (3 OTUs; 3 sequences) affiliated to SAR11 cluster, *Rhodospirillaceae* and *Rhodobacteraceae. Cyanobacteria* (8 OTUs; 27 sequences), *Actinobacteria* (5 OTUs; 6 sequences), *Marinimicrobia* (3 OTUs; 7 sequences), *Firmicutes* (2 OTUs; 13 sequences) to *Bacillus* sp., *Acidobacteria* (2 OTUs each; 6 sequences), one OTU each belonged to *Betaproteobacteria* and *Verrucomicrobia* sp. respectively (Fig 5c.2a and 5c.2b).

Fig 5c.2a

Fig 5c.2b

Figure 5c.2: Neighbor joining phylogenetic tree showing evolutionary relationship of phylotypes based on 16S rRNA gene sequences off Kochi during SIM: Spring Inter-Monsoon. Bootstrap analysis was performed with 1000 replicates and values > 50 % are indicated at the nodes. Scale bar represents the nucleotide substitution percentage.

a) Gammaproteobacteria, Alphaproteobacteria and Marinimicrobia

b) Cyanobacteria, Firmicutes, Betaproteobacteria, Acidobacteria, Verrucomicrobia and Actinobacteria

131

SuM: 25 OTUs were comprised of *Gammaproteobacteria* (10 OTUs; 38 clones) belonging to *Pseudomonas aeruginosa, Alteromonas, Idiomarina, Thalassomonas, Oceanobacter, Halomonas* sp. respectively and uncultured *gamma proteobacterium*. *Alphaproteobacteria* (5 OTUs; 11 sequences) belonged to *Rhodospirillales, Nitratireductor Sphingobium* and *Erythrobacter*. *Deltaproteobacteria* (3 OTUs; 6 sequences) belonging to SAR 324 cluster and uncultured *delta proteobacterium, Cyanobacteria* (1 OTU; 33 sequences), *Marinimicrobia* (3 OTUs; 4 sequences), *Chloroflexi* (2 OTUs; 5 sequences) and *Betaproteobacteria* (1 OTU; 3 sequences) belonging to *Burkholderia* sp. (Fig 5c.3).

FIM: Phylogenetic analyses showed that 58 OTUs were comprised of 20 OTUs out of 35 clones belonged to *Gammaproteobacteria* affiliated to SAR86 cluster, *Alteromonas, Pseudoalteromonas, Thalassomonas, Vibrio, Idiomarina*, *Methylophaga* and *Halomonas* sp. respectively. 15 OTUs; 20 sequences belonged to *Alphaproteobacteria* affiliated to SAR11 cluster, *Rhodobacteraceae, Donghicola eburneus, Sphingobium yanoikuyae, Nautella, Mesorhizobium* and *Erythrobacter, Deltaproteobacteria* (2 OTUs; 3 sequences) belonging to SAR324 cluster, *Betaproteobacteria* (3 OTUs; 9 sequences) each belonging to *Burkholderia* sp*., Cyanobacteria* (6 OTUs; 16 sequences) *Marinimicrobia* (3 OTUs; 3 sequences), *Acidobacteria* (2 OTUs; 3 sequences), *Firmicutes* (2 OTUs; 2 sequences) belonging to *Bacillus* sp*., Actinobacteria, Chloroflexi* and *Verrucomicrobia* (1 OTU; 2 sequences each), and one OTU each belonging to*, Planctomycetes* and *Bacteroidetes* (Fig 5c.4a, 5c.4b and 5c.4c).
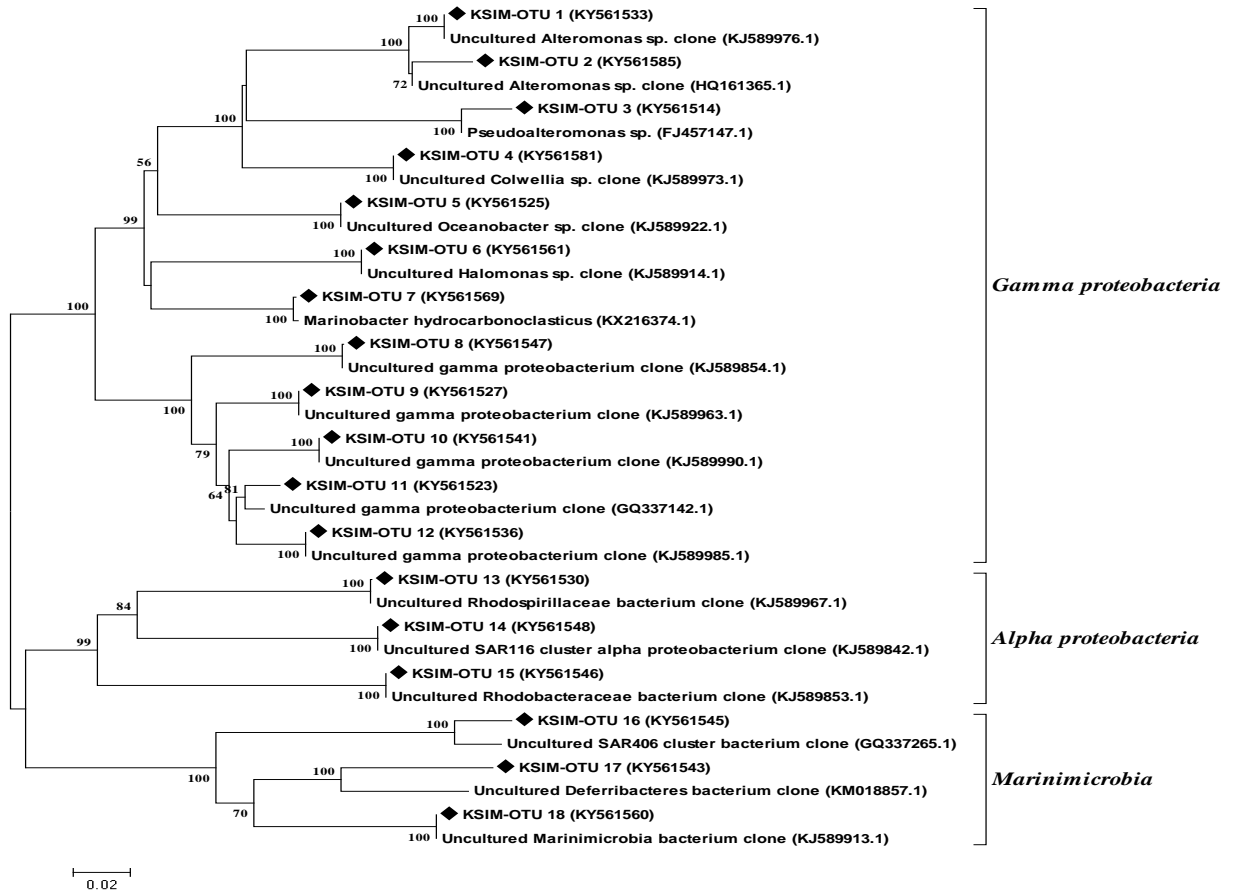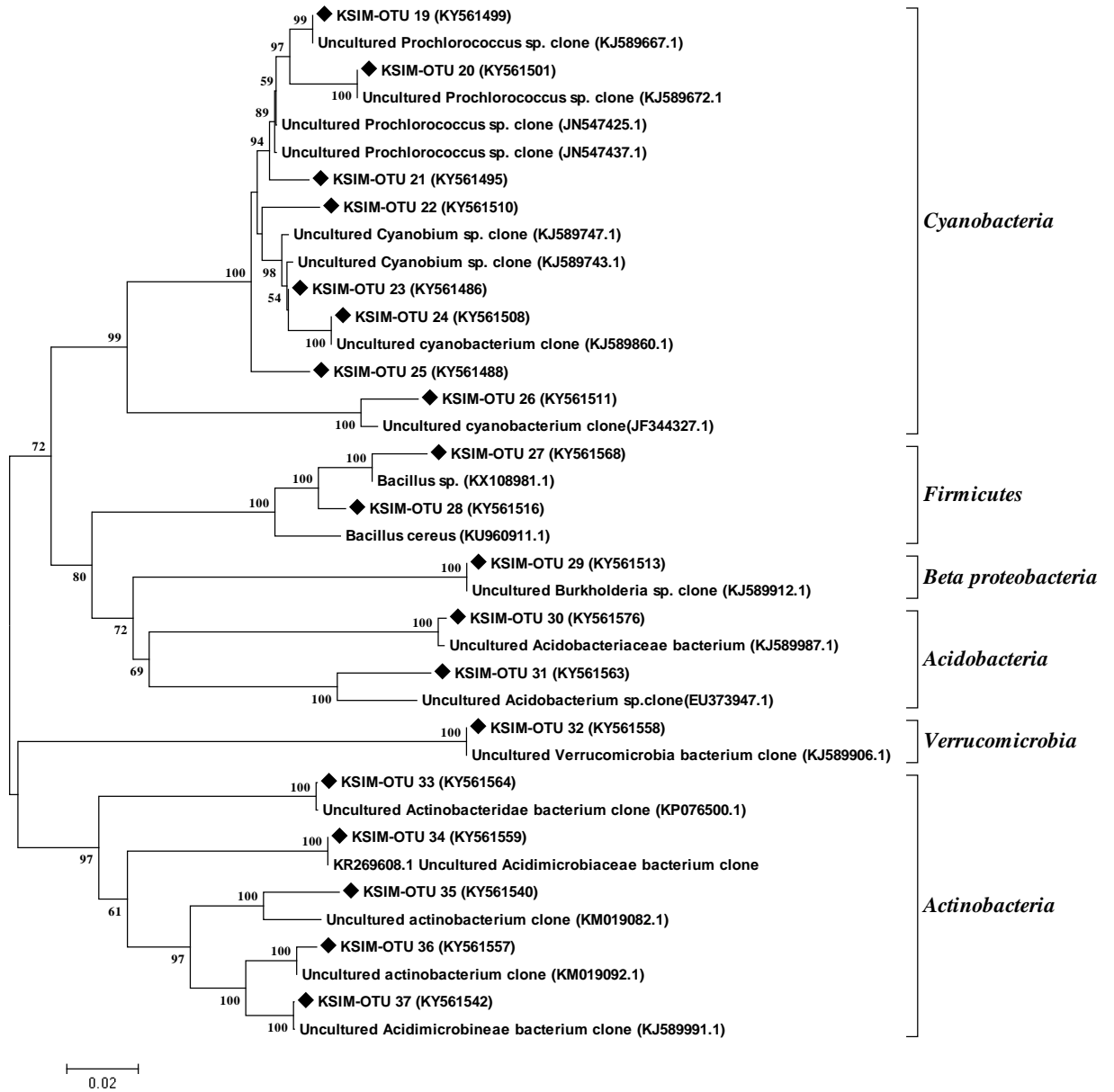
Fig 5c.3: Neighbor joining phylogenetic tree showing evolutionary relationship of phylotypes based on 16S rRNA gene sequences off Kochi during SuM: Summner monsoon. Bootstrap analysis was performed with 1000 replicates and values > 50 % are indicated at the nodes. Scale bar represents the nucleotide substitution percentage.

Fig 5c.4a

Fig 5c.4b

135

100 ■ KFIM-OTU 44 (KT361258)

Uncultured Acidobacteriales (JN886883.1)

Acidobacteria

50

■ KFIM-OTU 45 (KT361296)

100 Acidobacterium sp. (HQ675616.1)

■ KFIM-OTU 46 (KT361235)

100 Uncultured Chloroflexi bacterium (KJ589833.1)

Chloroflexi

86 ■ KFIM-OTU 47 (KT361260)

Bacillus subterraneus (KY202702.1)

99 ■ KFIM-OTU 48 (KT361316)

97 Bacillus sp. (KX021851.1)

Firmicutes

99 ■ KFIM-OTU 49 ()KT361301

Uncultured actinobacterium (KR269608.1)

Actinobacteria

■ KFIM-OTU 50 (KT361276)

100 Uncultured Verrucomicrobiales(EU361336.1)

Verrucomicrobia

■ KFIM-OTU 51 (KT361267)

98 Uncultured planctomycete (JX240979.1)

Planctomycete

100 ■ KFIM-OTU 52 (KT361305)

Uncultured Marinimicrobia (KJ589710.1)U

83

■ KFIM-OTU 53 (KT361269)

99 Uncultured Marinimicrobia (KJ589871.1)

60

■ KFIM-OTU 54 (KT361271)

100 Uncultured Marinimicrobia (KR269693.1)

Marinimicrobia

100 ■ KFIM-OTU 55 (KT361272)

Uncultured Flavobacteria (HQ433384.1)

Bacteroidetes

81 ■ KFIM-OTU 56 (KT361291)

Uncultured Burkholderia sp. (KJ589682.1)

65

■ KFIM-OTU 57(KT361292)

■ KFIM-OTU 58 (KT361288)

100

95 Uncultured Burkholderia sp. (KJ589688.1)

Uncultured Burkholderia sp. (KJ589680.1)
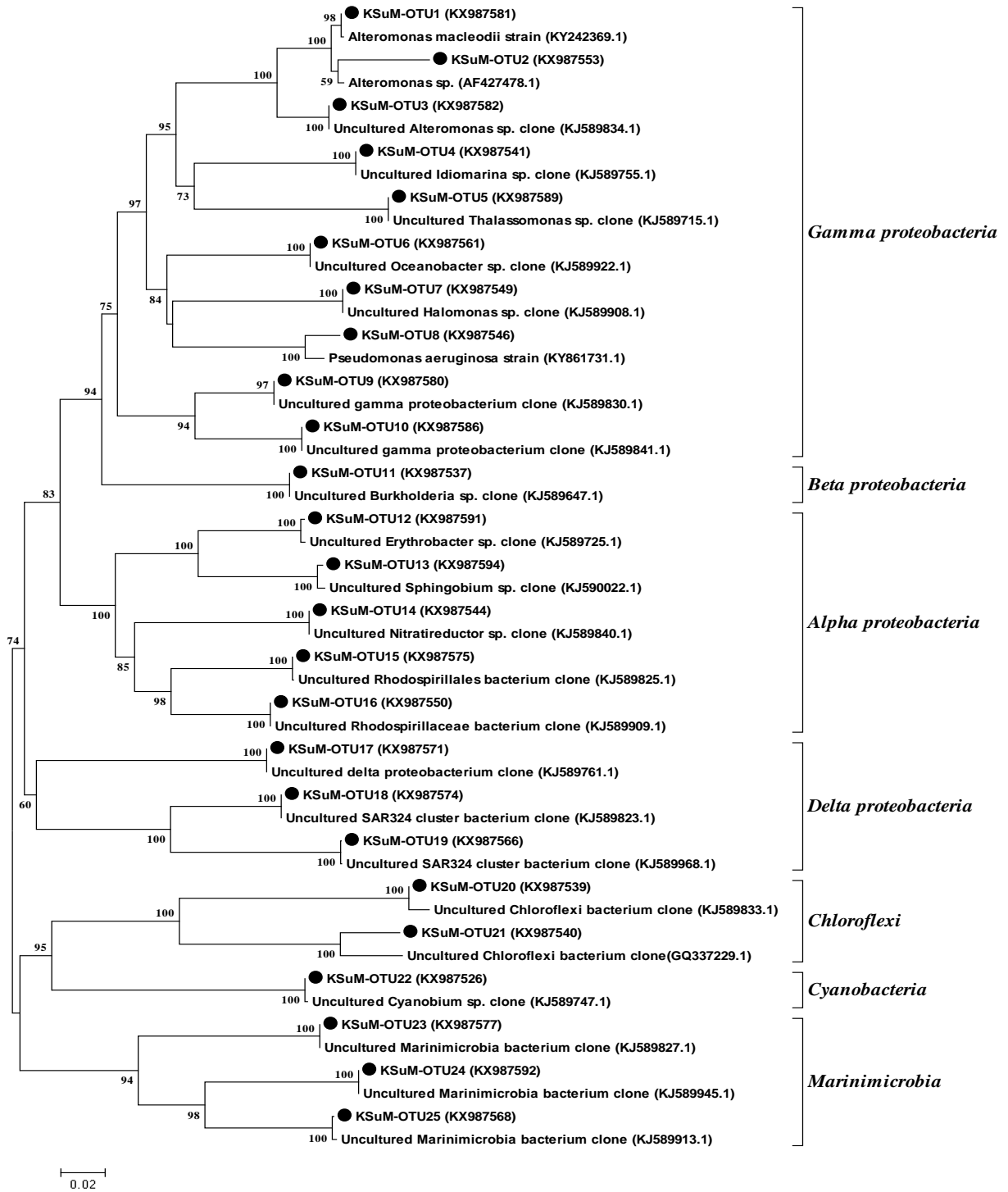
Beta proteobacteria

0.02

Fig 5c.4c

Figure 5c.4: Neighbor joining phylogenetic tree showing evolutionary relationship of phylotypes based on 16S rRNA gene sequences off Kochi during FIM: Fall Inter-Monsoon. Bootstrap analysis was performed with 1000 replicates and values > 50 %are indicated at the nodes. Scale bar represents the nucleotide substitution percentage.
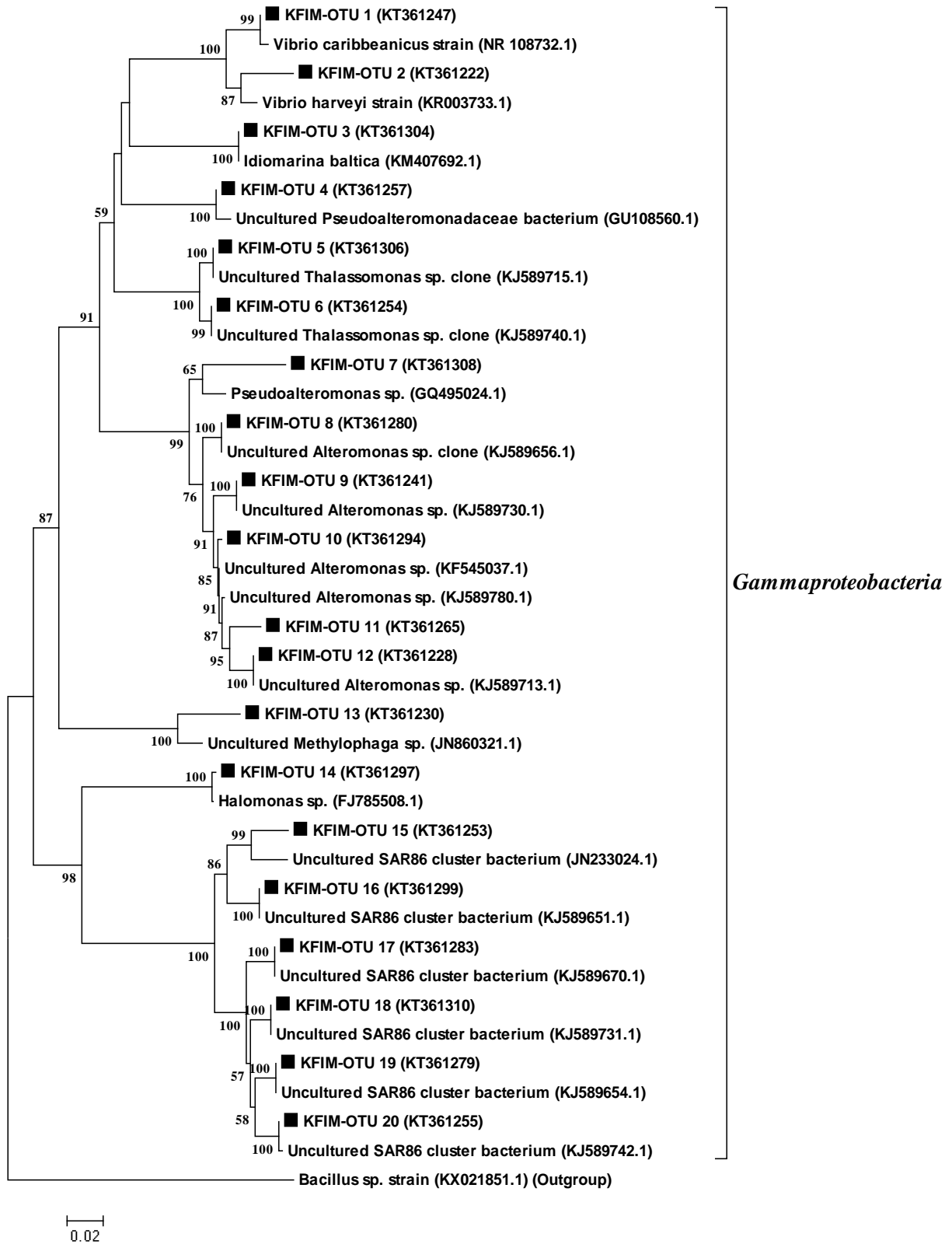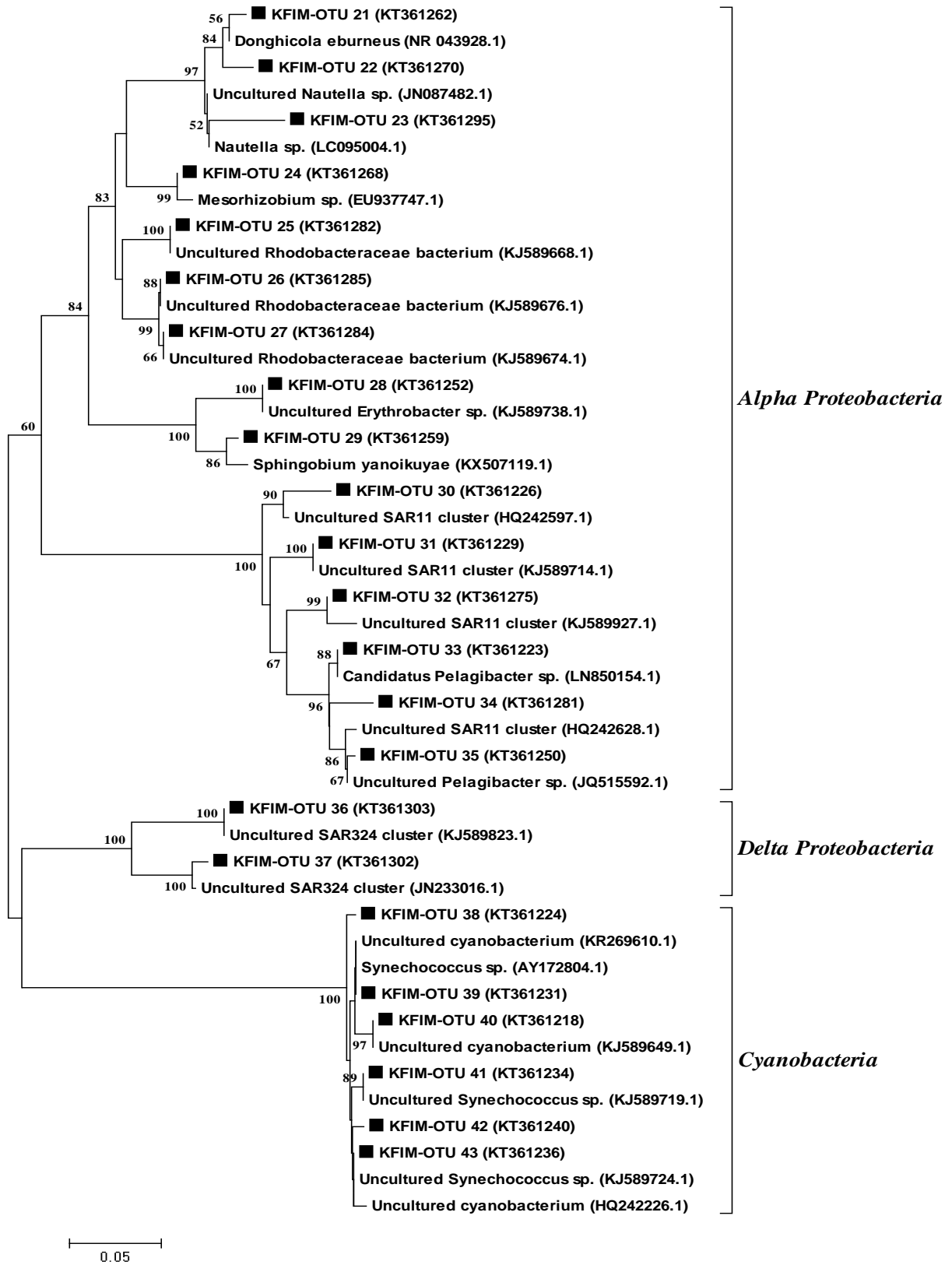
a) Gammaproteobacteria
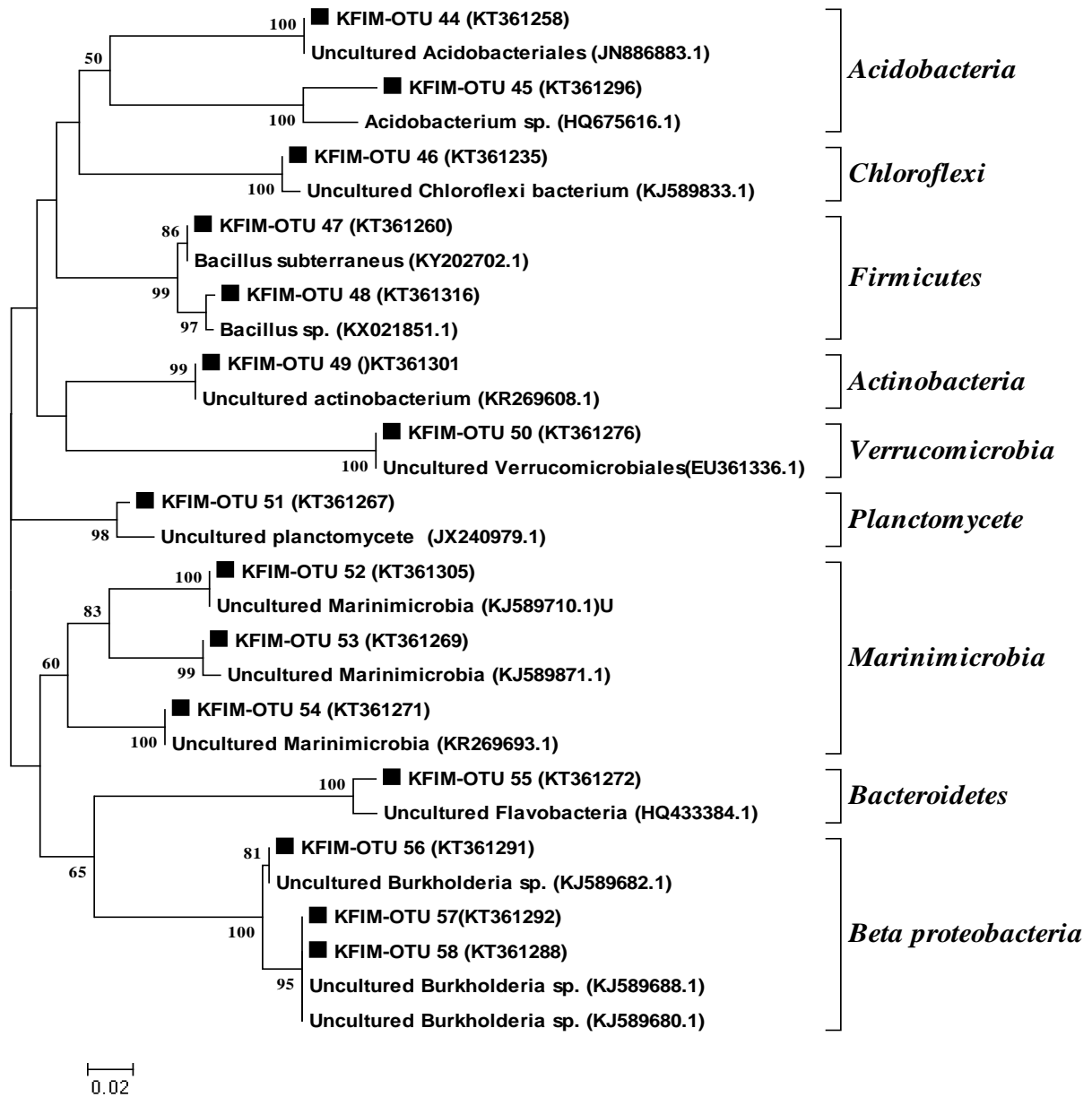
b) Alphaproteobacteria, Deltaproteobacteria and Cyanobacteria

c) Acidobacteria, Chloroflexi, Firmicutes, Actinobacteria, Verrucomicrobia, Planctomycetes, Marinimicrobia, Bacteroidetes and Betaproteobacteria

5c.3.3 Statistical analysis of 16S rRNA clone libraries

The Shannon and Simpson diversity indices were the highest during FIM (3.81 and 0.02, respectively) followed by SIM (3.33 and 0.04, respectively) and the lowest during SuM (2.73 and 0.09, respectively). Chao index also was higher in FIM in comparison to SIM and SuM. The estimated coverage values ranged from 63% to 87% (Table 5c.1). At equal sampling effort the rarefaction analysis showed that more ribogroups were found during FIM and reached near to saturation during SIM and SuM (Fig 5c.5).

Table 5c.1: Distribution of ribogroups, diversity indices and coverage of OTUs during different seasons from coastal waters of the eastern Arabian Sea Off Kochi

Diagnostic	SIM	SuM	FIM
No. of sequences	100	100	100
No. of OTUs	37	25	58
Shannon's index	3.33	2.73	3.81
Simpson's index	0.04	0.09	0.02
Chao1	98	46.5	102.4
Good's coverage (%)	72	87	63

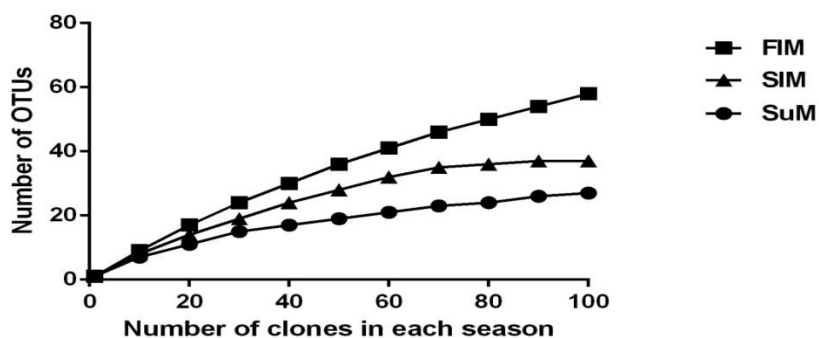**Fig 5c.5:** Rarefaction curves of operational taxonomic units (OTU) of 16SrRNA sequences obtained in each season.

DISCUSSION

5.4 <u>Discussion</u>

The physical and biogeochemical characteristic of West coast of India (WCI) is described in Chapter 3. The culture-independent phylogenetic analyses undertaken for this study is useful for understanding the bacterial roles in marine environments. *Alphaproteobacteria, Gammaproteobacteria* and *Cyanobacteria* were the predominant phylum observed along the WCI, besides these groups, members of *Betaproteobacteria, Deltaproteobacteria, Acidobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Verrucomicrobia, Planctomycetes, Chloroflexi* and *Marinimicrobia* were also present. Culture-dependent analysis from the WCI (Chapter 3) revealed that the presence of *Gammaproteobacteria*, *Alphaproteobacteria*, *Bacteroidetes*, *Firmicutes* and *Actinobacteria*. Former five phylogenetic groups were also obtained in bacterial 16S rRNA gene metagenomic clone library analysis. However, many uncultured bacteria recovered from the same regions belonged to more number of phyla.

5.4.1 Comparison of 16S rRNA gene clones with other oxygen deficient/minimum zones

Analysis of 16S rRNA gene clones in this study revealed that the bacterial community found in the study area resembled bacterial community composition in open-ocean and coastal oxygen minimum zones, enclosed or semi-enclosed euxinic basins (including the Northeast subarctic Pacific, the eastern tropical South Pacific, the Namibian upwelling, Saanich Inlet), glacial fjords and hypoxic estuaries (Stevens and Ullao, 2008; Divya et al. 2011; Jain et al. 2014; Wright et al. 2012; Spietz et al. 2015).

5.4.2 Bacterial community differences between off Goa, Mangalore and Kochi

No discernable difference was seen in the bacterial domains between off Goa, off Mangalore and off Kochi except for *Omnitrophica bacterium* found exclusively off Goa. Seasonal differences were quite distinct among the locations mentioned in results.

5.4.3 Roles of different bacterial domains

With the exception of few, most of the 16S rRNA gene sequences obtained from the clone libraries was closely related with the uncultured neighbours in the GenBank database.

Alphaproteobacteria

Rhodobacteraceae and SAR11 cluster were the dominant groups of the class *Alphaproteobacteria,* also reported from the ETSP OMZ (Stevens and Ulloa, 2008). SAR11 cluster are found to be abundant in OMZs and adapt to low oxygen, including genes for respiratory nitrate reductases (Tsementzi et al. 2016). Dimethylsulphoniopropionate (DMSP), an osmolyte produced by phytoplankton is considered as the major precursor of DMS in seawater, the oxidization of which leads to the formation of sulphate aerosols in the atmosphere that reflects solar radiation, and acts as cloud condensation nuclei upon which water vapour condenses and form cloud droplets (Shenoy et al. 2012). SAR11 cluster and members of the *Rhodobacteraceae* such as *Loktanella, Roseobacter sp*. are found to oxidize DMSP to DMS, which play a significant role in controlling the amount of incident radiation reaching the Earth's surface (Howard et al. 2006; Curson et al. 2008; Zeng et al. 2016) signifying their role in climate regulation. *Rhodospirillaceae* are mainly found in anaerobic environments and are reported to possess dsrAB gene involved in both the reductive and the

oxidative steps of the biogeochemical sulfur cycle (Thrash et al. 2016). Members of *Phyllobacteriaceae* such as *Nitratireductor aquibiodomus* and *Mesorhizobium* sp. are known to be denitrifiers (Labbé et al. 2004). *Sphingomonadales* has been recognized to have broad metabolic capacity (Miller et al. 2010) and are known to degrade aromatic compounds (Fredrickson et al. 1995). *Erythrobacter* is a known anoxygenic phototrophic bacterium contributing to the marine carbon cycling (Kolber et al. 2001).

Gammaproteobacteria

Alteromonas sp. was the dominant group of this class followed by *Vibrio* sp. *Alteromonas* are copiotrophic bacteria widely distributed throughout the marine environment, where they can play a notable role in the processing of dissolved organic carbon pools (López-Pérez et al. 2012), therefore it is present abundantly in organic rich waters. McCarren et al. (2010) indicated the involvement of *Idiomarina* sp. and *Alteromonas macleodii* in metabolizing semilabile high molecular weight dissolved organic matter to methanol or formaldehyde, and carbon dioxide. The methanol and/or formaldehyde produced could be further oxidized and incorporated by *Methylophaga* sp. Further, *Vibrio* sp. are normal residents in coastal waters and play an important role in biodegradation, nutrient regeneration and biogeochemical cycling (Cavallo and Stabili, 2004). Recent study by Li et al. (2013) demonstrated efficient denitrifying ability by *Marinobacter* sp., likewise, *Pseudomonas* sp. is one of the widely reported denitrifying heterotrophic bacteria, and it has been considered as a model system for the denitrification process (Lalucat, 2006; Rezaee et al. 2010). *Methylophaga* and *Pseudomonas* sp. are found to oxidize DMSP to DMS (Reisch et al. 2011; Zeng et al. 2016). *Marinobacter* sp. is reported to degrade the hydrocarbons and can use

nitrate as the terminal electron acceptor (Gauthier et al. 1992). *Thalassomonas* is involved in nitrate reduction (Jean et al. 2006). SAR 86 cluster have the capacity to utilize a range of polysaccharides and lipids (Dupont et al. 2012). *Halomonas* are reported to denitrify (González-Domenech et al. 2010).

Betaproteobacteria

The members of this phylum are reported to be predominant in the freshwater systems (Mueller-Spitz et al. 2009). Sequences related to *Burkholderia* sp were found in this study. They were reported earlier by Divya et al. (2011) from the organic–rich sediments underlying the oxygen-deficient waters in the eastern Arabian Sea. 16S rRNA gene clones affiliated to this phylum is common in coastal samples (Yeo et al. 2013). These findings imply that this group of bacteria have a freshwater origin and adapt to the coastal marine environment and the representative phylotype could transit between freshwater and marine habitats (Rappé and Giovannoni, 2003).

Deltaproteobacteria

These are reported to be abundant in OMZs, where they are implied to play important roles in linking the sulfur and nitrogen cycles (Canfield et al. 2010; Stewart et al. 2012; Wright et al. 2012). Sulfur oxidation was recently suggested to be a key physiological feature of SAR 324 (Sheik et al., 2014).

Marinimicrobia

Marinimicrobia bacteria are abundant in the oxygen minimum zones and are involved in partial denitrification (nitrate reduction) (Bertagnolli et al. 2017).

Cyanobacteria

Oxygen that is produced by *Cyanobacteria* as a result of oxygenic photosynthesis plays fundamental roles in the global biogeochemical cycling of nitrogen, sulphur, carbon (Sanchez-Baracaldo et al. 2005).

Actinobacteria

These are the substantial part of the bacterial community in the coastal waters of the Arabian Sea (Singh and Ramaiah, 2011).

Acidobacteria

These have been reported to be capable of nitrate and nitrite reduction in soils (Ward et al. 2009a; Kielak et. al. 2016) and in degradation of recalcitrant organic carbon sources (Quaiser et al. 2008). Their actual diversity, relative abundance, and ecological role in the oceans remain unknown.

Firmicutes

The members belonged to genus *Bacillus* was also reported from OMZ sediments Divya et al. (2011). Many different species of *Bacillus* (Verbaendert et al. 2011) are reported to be efficient denitrifiers.

Bacteroidetes

These contribute to <10 % of the community structure in marine environments and the family *Flavobacteraceae* is observed in oceans. These are abundant in nutrient-rich waters and are major utilizers of high-molecular-mass dissolved organic matter in marine ecosystems (Kirchman, 2002).

Verrucomicrobia

This group appears to be lower in marine environments compared with soil and are known to play significant ecophysiological and biogeochemical roles (Bergmann et al. 2011).

Planctomycetes

These which are also reported in marine hypoxic regions, are known to breakdown of sulfated heteropolysaccharides (Ye et al. 2015)

*Chloroflex*i

These are involved denitrification and carbon fixation (Yilmaz et al. 2015).

5.4.4 Relevance of the community to hypoxia and denitrification

Assessment of bacterial community composition is a requirement to recognize their possible functional roles. The present study is useful to recognize that bacterial community undergoes shifts temporally in response to environmental variables (temperature, dissolved oxygen, nitrate and nitrite). Bacteria were more diverse during FIM when suboxia is evident. As such, diverse groups of bacteria proliferate with the availability of higher quantities of

organic matter as a consequence of increased primary production due to elevated nitrate concentrations in particular during FIM (Singh and Ramaiah, 2011; Du et al. 2013). The high nutrient inputs from anthropogenic activities could also alter the taxonomic composition of bacterial community (Simonato et al. 2010). Dissolved oxygen (DO) has been proved to be capable of altering bacterial communities in many marine systems (Stevens and Ulloa, 2008; Zaikova et al. 2010). The low DO concentration could compress the habitat available to aerobic organisms forcing them to exploit alternative electron acceptors leading to denitrification resulting in changes of biogeochemical cycles (Naqvi et al. 2000; Rabalais et al. 2010).

5.4.5 Conclusion

Along the west coast of India, the bacterial communities respond rather strongly to changing seasonal environmental conditions with physiological adaptation or by shifts in the community composition. It is possible that these seasonal hydrographic conditions, in particular reduced oxygen, and their influence on bacteria, contributed significantly to the observed shifts both in diversity, type and predominance. These observations on the distribution of bacterioplankton in the seasonally hypoxic coastal waters are useful to recognize their importance in denitrification process in particular during hypoxic periods. A common observation was that the bacterial communities in the bottom waters were quite diverse from those at the surface, implying that the bacterial populations were affected by the prevailing physicochemical conditions in the water column.

QUALITATIVE AND QUANTITATIVE ANALYSES OF *Nar*, *Nir* AND *Nos* GENES

6.1 <u>Introduction</u>

Diverse microbial communities perform the biogeochemical transformation of biologically essential elements. Among such functions, the microbial ability to denitrify (i.e., removal of fixed nitrogen) is possessed by many taxonomic groups of bacteria (Braker et al. 2000; Jayakumar et al. 2004). Fixed nitrogen in its oxidized forms is used as an alternative electron acceptor to support respiration when oxygen is limiting. Denitrification is the sequential reduction of nitrate (NO_3^-) to nitrite (NO_2^-), to nitric (NO), to nitrous oxide (N_2O), and subsequently to dinitrogen gas N_2. In general, marine denitrification is widely reported from -and understood to occur in- low oxygen regions of the water column (Stevens and Ulloa, 2008; Ward et al. 2009; Bristow et al. 2017). The Arabian Sea oxygen minimum zone (OMZ), which covers only ~2 % of the global oceanic area, accounts for ~20 % of oceanic denitrification and, hence, has a significant role in marine nitrogen budget (Bange et al. 2005; Ward et al. 2009b). The oxygen deficiency in the intermediate layer of ~150-1000 m water column of this region (Bange et al. 2005; Jayakumar et al. 2009b), results from a high oxygen demand and poor reach of oxygen-rich Antarctic bottom water to these intermediate waters.

In addition to the perennial open-ocean OMZ, pronounced oxygen deficient conditions do develop seasonally along the southwest coast of India, in particular during the southwest monsoon season (June to October). The source of upwelled water is the poleward undercurrent. Intense upwelling due to local and remote forcing by monsoon winds entrain low-oxic and high nutrient waters to the photic layers (Shetye et al. 1990). During this period, increased respiration of locally produced organic matter in conjunction with strong stratification (Shetye and Gouveia, 1998) lead to intense denitrification (Jayakumar et al. 2004), which in turn leads to production and accumulation of the potent greenhouse gas, N_2O.

Seasonal oxygen deficiency is acknowledged to be detrimental to coastal ecosystems adversely affecting fish, benthic fauna, and crustaceans through altered trophic efficiency/energy transfer (Levin et al. 2009; Rabalais et al. 2010; D'Silva et al. 2012). In the overall, the Arabian Sea denitrification is reported to account for about 30% of the oceanic fixed nitrogen loss. This region is also known to contribute to the highest N_2O flux to the atmosphere (Naqvi et al. 2000; Bange et al. 2005). With the reversal of coastal circulation, the water column becomes oxygenated again in November and oxidation of organic matter, apparently is oxygen/aerobic respiration dominated (Codispoti et al. 2001). Denitrifiers comprise a polyphyletic group of mostly heterotrophic microorganisms which share the ability to denitrify among distantly related genera. Whether perennial as in the Arabian Sea OMZ or seasonal as is along the west coast of India, the process of denitrification is reported to be performed by such diverse groups of bacteria capable of denitrifying by switching between aerobic and NO_3^- dependent modes of respiration (Zumft, 1997; Yu et al. 2014). The enzymes involved in denitrification are reductases of nitrate, nitrite and nitrous oxide encoded respectively by *nar*, *nir* and *nos* genes.

The first step in the microbially mediated denitrification process is the reduction of NO_3^- to NO_2^- catalyzed by a membrane-bound NO_3^- reductase (*nar*) or periplasmic NO_3^- reductase (*nap*), encoded by the *narG* or the *napA* genes. Denitrifying bacteria are known to possess one or both of these reductases (i.e., *narG* and *napA*), wherein *narG* is considered to be more extensive and representative (Deiglmayr et al. 2004; Smith et al. 2007; Reyna et al. 2010). In the second step, NO_2^- reduces to NO catalyzed by two functionally and physiologically equivalent types of NO_2^- reductases, either a cytochrome cd1 (encoded by *nirS*) or a Cu-containing enzyme (encoded by *nirK*; Glockner et al. 1993). NO reduces to the

intermediate N_2O some of which ultimately escapes to the atmosphere. The reduction of N_2O to N_2 is the last step, which, as reported by Throbäck et al. (2004), is catalyzed by N_2O reductase encoded by the *nosZ* gene present in the periplasm. As Ferguson (1994) suggested, it is likely that the transcription and subsequent elaboration/build-up of denitrifying enzymes do not begin until oxygen levels drop down and, there is adequate availability of NO_3^- / NO_2. The polyphyletic origin of denitrification makes 16S rRNA gene-based approach impossible to study the denitrifiers present; therefore research has focused on targeting functional marker genes for recognizing the denitrification process (Jones et al. 2008). Over the past decade, *narG*, *nirS,* and *nosZ* genes have been widely used to describe denitrifier communities (Braker et al. 2000; Scala and Kerkhof et al. 1998; Throbäck et al. 2004; Henry et al. 2006; Bru et al. 2007).

The present study aimed to detect and quantify three genes (*nar*, *nir,* and *nos*) involved in denitrification. As Ward et al. (2009b) and Lam et al. (2011) highlight, estimation of qPCR-based abundances of denitrifying bacteria ought to yield reliable estimates of their contributions to the total microbial abundance. Such essential knowledge would prove useful in understanding the response of microbial communities due to seasonally changing environmental conditions, in regions such as the west coast of India which experiences seasonal hypoxia. The overall objectives were i) to quantify the abundance of denitrifying functional genes (*narG, nirS,* and *nosZ*) in the water column of the coastal region of the Arabian Sea and ii) to investigate the bacterial diversity of these denitrifying functional genes.

6.2 <u>Materials and methods</u>

6.2.1 Sample Collection

Location of the study area and its details are specified in Chapter 3, section 3.2.2. For phylogenetic analyses, seawater collected from G9, M8 and K3 during all three seasons was used.

6.2.2 Extraction of Metagenomic DNA

Extraction of metagenomic DNA is described in section 5.2.2. The same DNA samples were used for this study as well.

6.2.3 PCR amplification of denitrifying functional genes *narG, nirS,* and *nosZ*

PCR amplification of denitrifying genes was performed in 50 µl volume using Taq PCR reaction mix as per manufacturer's instructions (Sigma-Aldrich) using primers listed in Table 6.1. Amplicons of *narG* and *nirS* and were obtained according to the protocol of Bru et al. (2007) and Throbäck et al. (2004). After the initial denaturation at 95 °C for 10 min, 6 cycles of 95 °C for 15 s, 63 °C for 30 s, and 72 °C for 30 s of touchdown was carried out, with a 1 °C step down in annealing temperature of each cycle. Followed by 35 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 90 s and a final extension at 72 °C for 10 min, *nosZ* amplicons were obtained using primer sets developed by Kloos et al. (2001). The reaction was initially denatured at 94 °C for 2 min; followed by 34 cycles of 94 °C for 40 s, 57 °C for 40 s, and 72 °C for 40 s and a final extension step at 72 °C for 8 min. The PCR products were analyzed on 1.5 % (w/v) ethidium bromide-stained agarose gel to ensure that the correct size fragment was amplified.

6.2.4 Construction of clone libraries

The amplicons of each three above functional gene products were purified using UltraClean® PCR Clean-Up Kit. They were then ligated and cloned using a TOPO TA cloning kit. The PCR product was ligated into a pCR® 4-TOPO plasmid vector and cloned into TOP10 competent *Escherichia coli* cells (Invitrogen) according to the manufacturer's instructions. Transformants were selected on Luria-Bertani agar plates containing ampicillin (100 μg ml^{-1}) and X-Gal (5-Bromo-4-chloro-3-indolyl-D-galactopyranoside) (20 μg ml^{-1}). White colonies were screened by colony PCR using the vector primers M13F (5' GTAAA ACGAC GGCCA GT3') and M13R (5' CAGGA AACAG CTATG AC3') for checking the positive transformants.

6.2.5 Sequencing, phylogenetic tree construction and statistical analysis

The clones were sequenced by using an ABI 3130XL genetic analyzer (Applied Bio-Systems). The sequences obtained were edited with the software Vecscreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/), and checked for chimeras using DECIPHER's (http://decipher.cee.wisc.edu/). Gene sequences were aligned and grouped using the MOTHUR program at a 97 % cut-off level. Only one representative sequence from each OTU was taken for phylogenetic analyses. These representative sequences were checked for their similarity with sequences in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov). Sequences from this study were aligned with matching ones in the GenBank database using ClustalX 1.83 (Thompson et al. 1994). The Neighbor-joining method was followed to construct the tree using the software, MEGA4 and the maximum likelihood method was used for calculating the evolutionary distance (Tamura et al. 2007). Bootstrap analysis (1000

replicates) was done to confirm the reliability of the phylogenetic tree. From all libraries, the rarefaction analysis, richness and non-parametric diversity index (Chao I) and coverage were carried out using the MOTHUR program (Schloss et al. 2009).

6.2.6 Nucleotide sequence accession numbers

Sequences were submitted to GenBank and accession numbers assigned to our submission are KX034220-KX260230, KX303594-KX303723, KX907784-KX907837, KY354437-KY354512, KU862040-KU862166, KX982367-KX982499, KY465775-KY465814, KX290694-KX290695, KX344419-KX344426, KX354182-KX354201, KX387811-KX387840, KX398618-KX398647, KX495676-KX495685, KX581699-KX581718, KX639726-KX639736, KX655567-KX655573, KX768016-KX768029, KX832794-KX832813 and KX871698-KX871705

Table 6.1: Primers used for PCR amplification of denitrification genes

Target gene	Primer	Primer sequence(5´-3´)	Product size (bp)	Reference
NarG	*NarG-F*	TCGCCSATYCCGGCSATGTC	174	Bru et al. (2007)
	NarG-R	GAGTTGTACCAGTCRGCSGAYTCSG		
NirS	*NirScd3aF-F*	AACGYSAAGGARACSGG	425	Throback et al. (2004)
	NirSR3cd-R	GASTTCGGRTGSGTCTTSAYGAA		
NosZ	*NosZ1840-F*	CGCRACGGCAASAAGGTSMSSGT	267	Henry et al. (2006)
	NosZ2090-R	CAKRTGCAKSGCRTGGCAGAA		
	NosZ-F	CG(CT)TGTTCMTCGACAGCCAG	700	Kloos et al.(2001)
	NosZ-R	CATGTGCAGNGCRTGGCAGAA		

6.2.7 Quantitative analysis of *narG*, *nirS* and *nosZ* genes

For quantitative analyses, the DNA standards used consisted of plasmids carrying the appropriate target genes that were previously sequenced to confirm their identity. Serial dilutions (Ten-fold) of a known copy number of the plasmid DNA containing the desired gene were subjected to qPCR assay in triplicate to generate an external standard curve. Standard curves for *narG*, *nirS* and *nosZ* assays were generated by plotting the threshold cycle values versus log10 of the gene copy numbers. For each standard curve, the slope, *y*-intercept, and coefficient of determination (r^2) were determined. The amplification efficiency (E) was calculated using the equation E= $(10^{1/slope} - 1)$ x 100

The abundance of *narG*, *nirS* and *nosZ* genes was quantified in triplicate by using the ABI 7500 Real-Time PCR system (Applied Biosystems, USA). The same primers used to construct the clone libraries were used to quantify *narG* and *nirS*, whereas *nosZ* abundance was quantified using primer set NosZ 1840-F and NosZ 2090-R developed by Henry et al. (2006) (Table 1). Each reaction was performed in 20-µl volume containing 4 µl of DNA template having concentration of 12-15 ng/µl, 0.5 µl 10 pm of each primer and 12.5 µl of 5×qARTA Green qPCR Mix (AXYGEN, USA). The PCR temperature program begun with an initial denaturation for 15 min at 95°C, followed by a total of 40 cycles of 15 s at 95°C, and annealing/extension for 1 min at 60°C. The copy number of the target genes (*narG*, *nirS* and *nosZ*) was calculated via comparison to standard curves.

6.3 Results

6.3.1 PCR amplification and abundance of *narG, nirS* and *nosZ* genes

All the DNA samples were PCR amplified using the 16S rRNA primers to confirm the presence of amplifiable DNA from all samples. Amplification of *narG* gene was positive at near bottom waters during SuM and FIM at the three locations, while subsurface amplification was evident off Kochi. A Similar pattern was observed in case of *nirS* gene during SuM and FIM, except for no amplification off Mangalore during FIM. However, *nosZ* gene amplification was evident only in bottom depths during SuM and FIM. There was no amplification using the above genes during SIM.

All the standard curves, generated using plasmid containing the cloned *narG, nirS*, and *nosZ* genes, correlated strongly with efficiencies of $r^2 > 0.99$. They were used as the references to calculate the concentrations of these genes in the environmental DNA samples. The efficiency of PCR amplification of *narG, nirS*, and *nosZ* genes was 92%, 80%, and 85%, respectively. The abundance of these functional genes was different and widely varying between the sampling locations. The highest abundance was of *narG* gene. The abundance of *narG*, *nirS*, and *nosZ* genes was more in the low oxygen-waters near-bottom as well as with the intensifying suboxia during FIM.

The range of *narG* copy numbers off Goa during the times of low oxygen concentrations was 3.9 to 7.9 x 10^7 copies L^{-1}. Similarly, the *narG* copy numbers were in the range of 1.5 to 3.9 x 10^7 copies L^{-1} off Mangalore and 1.5 to 3.9 x 10^7 copies L^{-1} off Kochi. The range of *nirS* copy numbers off Goa during the times of low oxygen concentrations was 0.14 to 0.21 x 10^6 copies L^{-1}. Likewise, the *nirS* copy numbers were in the range of 0.001 to 0.23 x 10^6 copies L^{-1} off Mangalore and 0.006 to 0.30 x 10^6 copies L^{-1} off Kochi. The range of

nosZ copy numbers off Goa during the times of low oxygen concentrations was 2.80 to 2.90 x 10^6 copies L^{-1}. Likewise, the *nosZ* copy numbers were in the range of 0.75 to 1.70 x 10^6 copies L^{-1} off Mangalore and 0.30 to 0.45 x 10^6 copies L^{-1} off Kochi (Fig 6.1).
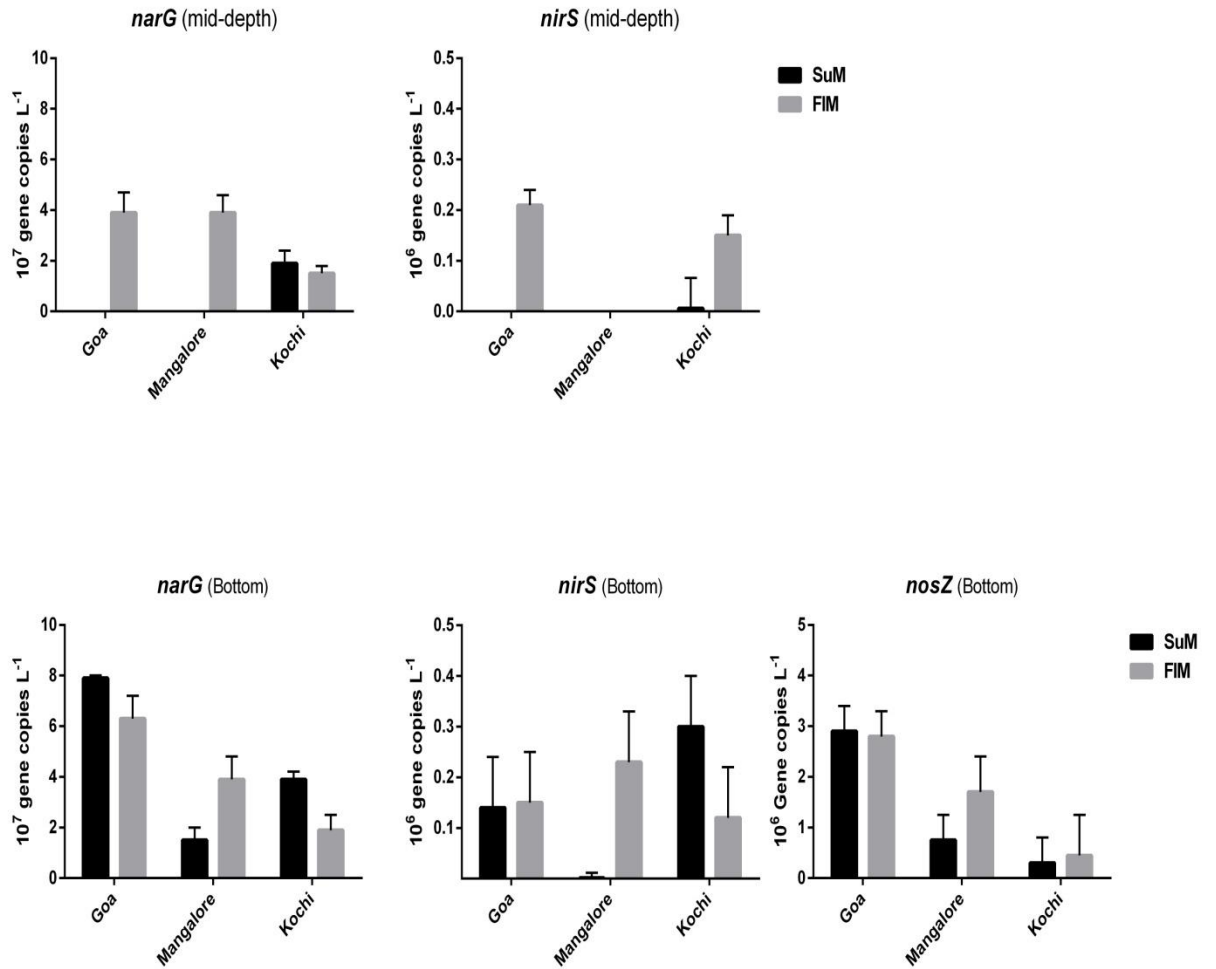

Fig.6.1: Seasonal variation in Copy numbers (L^{-1}) of denitrifying genes at sampling locations along the west coast of India

6.3.2 Phylogenetic analysis of denitrifying functional genes

Clone libraries were constructed from all the DNA extracts showing positive amplification for the functional genes (*narG, nirS,* and *nosZ*) from Goa, Mangalore, and Kochi. All three genes were mostly detected from low oxygen near bottom samples during SuM and FIM.

narG: A total of 300 non chimeric sequences obtained during SuM (120 sequences) and FIM (180 sequences) split into three clusters in the phylogenetic tree. 86.3% sequences grouped into Cluster I with clones affiliating to *Marinobacter, Shewanella*, *Halomonas*, *Pseudomonas, Pectobacterium, Stenotrophomonas* sp. belonging to *Gammaproteobacteria* and uncultured representatives from paddy soil and saline alkaline soil. Cluster II (10% sequences) were related to *Burkholderia, Delftia* and *Alicycliphilus* sp. belonging to *Betaproteobacteria*. Cluster III (2% sequences) belonged to *Brucella*, *Methylobacterium* and *Ochrobactrum* sp. affiliating to *Alphaproteobacteria* (Fig.6.2).

nirS: 270 sequences obtained in SuM and FIM (120 and 150 each) categorized into two clusters in the phylogenetic tree. Cluster I (90% sequences) related to cultured representatives *Pseudomonas stutzeri*, *Alcaligenes faecalis*, *Kocuria varians*, *Herbaspirillum* and *Pseudogulbenkiania* sp. While cluster II (10% sequences) comprised of environmental samples (Fig 6.3).

nosZ: In total 200 sequences, 100 each from FIM and SuM could be classified into three clusters in the phylogenetic tree. I (3% sequences) was related to *Pseudomonas aeruginosa* belonging to *Gammaproteobacteria*. II (4% sequences) clustered were related to *Achromobacter cycloclastes, Rhodoferax ferrireducans* and *Herbaspirillum* sp. affiliating to

Betaproteobacteria. III (93% sequences) contained clones related to *Bradyrhizobiaceae, Shinella zoogloeoides, Rhodopseudomonas palustris, Dinoroseobacter shibae, Ruegeria pomeroyi, Rhodobacter sphaeroides f.* sp. *denitrificans, Paracoccus Mesorhizobium, Azospirillum* and *Sinorhizobium* sp. belonging to *Alphaproteobacteria* (Fig 6.4).

53 ■ G-FIM-Bottom-165 (198)
65 ● M-SuM-Bottom-33 (22)
100 Marinobacter hydrocarbonoclasticus(FO203363.1)
Marinobacter sp.(JQ745300.1)
67
■ G-FIM-Bottom-164(3)
100 Shewanella woodyi (CP000961.1)
94
87 ● M-SuM-Bottom-48(4)
■ K-FIM-Mid-298(4)
100 Halomonas halodenitrificans(AB076402.2)
96 Halomonas chromatireducens (CP014226.1)
97
97 ■ G-FIM-Bottom-175 (10)
99 Pseudomonas denitrificans (CP004143.1)
● K-SuM-Bottom-118(7)
98 Pseudomonas stutzeri(CP003725.1)
98 52
● G-SuM-Bottom-183(5)
100 Pectobacterium wasabiae (CP015750.1)
72 saline alkaline soil(EU495777.1)
93 ■ M-FIM-Mid-181 (2)
● K-SuM-Bottom-253(1)
99 Stenotrophomonas maltophilia (CP002986.1)
99
89 Paddy soil(FJ209573.1)
■ M-FIM-Mid-200 (1)
93 ● K-SuM-Mid-70(1)
97 ■ K-FIM-Mid-241(1)

Cluster I

■ K-FIM-Mid-128 (2)
100 ● M-SuM-Bottom-223 (3)
Alicycliphilus denitrificans(CP002657.1)
51 ■ K-FIM-Mid-143 (2)
100 ■ K-FIM-Bottom-176 (6)
Delftia sp.(CP018101.1)
90 99 ■ K-FIM-Bottom-158 (6)
Burkholderia ambifaria (CP009800.1)
100 99 ■ K-FIM-Bottom-157 (4)
Burkholderia pseudomallei (CP016912.1)
80 Burkholderia sp (CP013387.1)
69 Burkholderia fungorum(CP010027.1)
● G-SuM-Bottom-184 (6)
Burkholderia glumae(CP009434.1)

Cluster II

100 ■ G-FIM-Bottom-56(5)
Methylobacterium nodulans (CP001349.1)
100 ■ K-FIM-Mid-135(2)
Ochrobactrum anthropi (CP008819.1)
97 ● G-SuM-Bottom-198 (5)
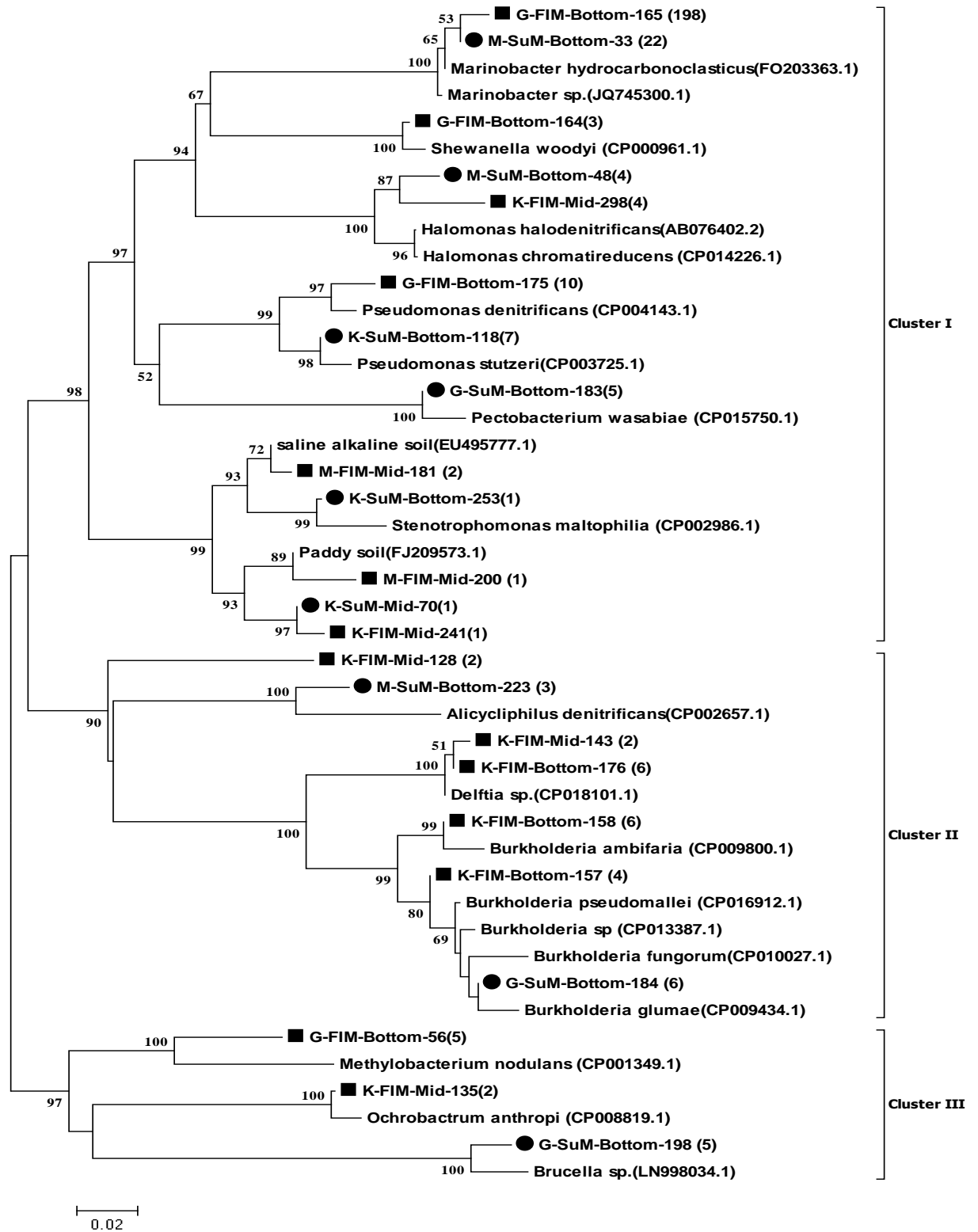100 Brucella sp.(LN998034.1)

Cluster III

0.02

Fig.6.2: Neighbor joining tree generated from alignments of *narG* sequences from the three different locations during SuM and FIM, and their representative references derived from the

157

Genbank. Tree topology was inferred by using 1000 bootstrap iterations. Accessions numbers are shown in parentheses. Bootstrap values >50% are shown at each node. Scale bars represent the nucleotide substitution percentage. Clone designations are shown by symbols: ●SuM , ■FIM and letters as G, M and K corresponding to the locations (Goa, Mangalore and Kochi) from where the clones were derived.

Fig.6.3: Neighbor joining tree generated from alignments of *nirS* sequences from the three different locations during SuM and FIM, and their representative references derived from the Genbank.Tree topology was inferred by using 1000 bootstrap iterations. Accessions numbers are shown in parentheses. Bootstrap values > 50% are shown at each node. Scale bars represent the nucleotide substitution percentage. Clone designations are shown by symbols: ●SuM , ■FIM and letters as G, M and K corresponding to the locations (Goa, Mangalore and Kochi) from where the clones were derived.
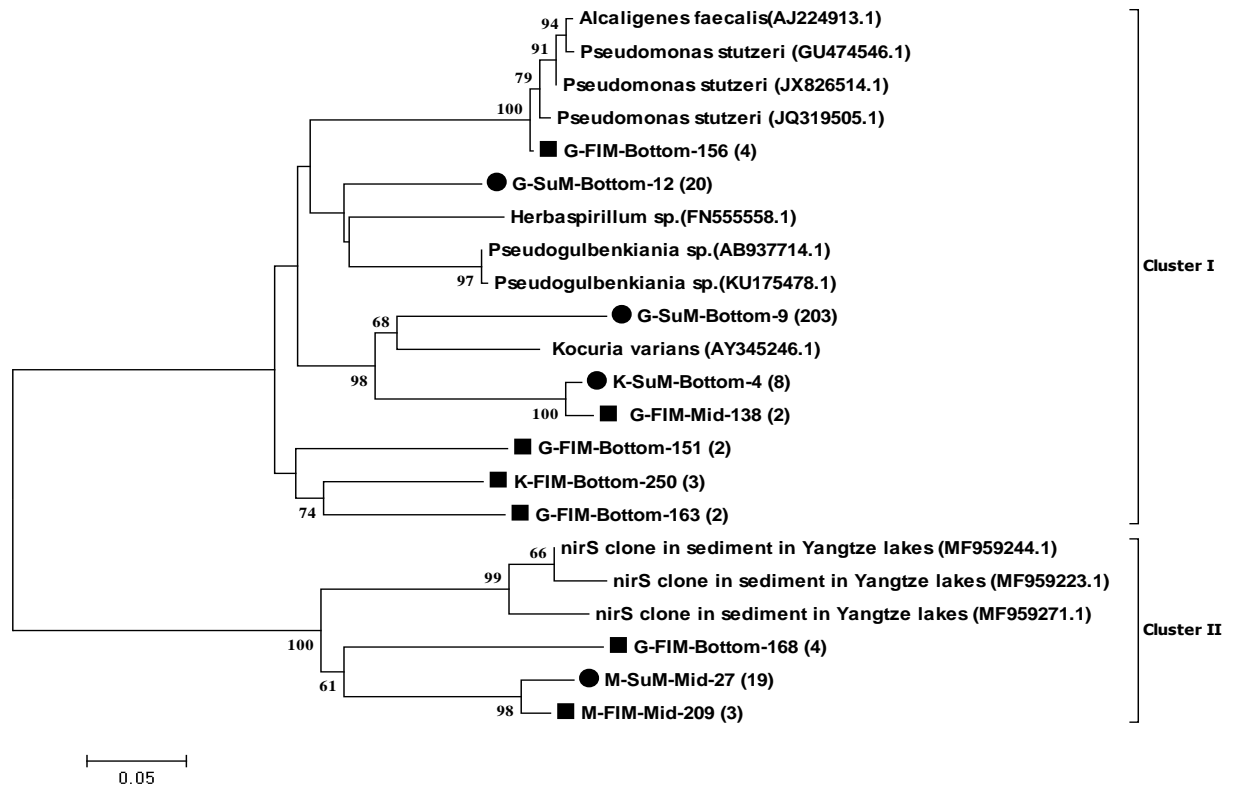
Fig.6.4: Neighbor joining tree generated from alignments of *nosZ* sequences from the three different locations during SuM and FIM, and their representative references derived from the Genbank. Tree topology was inferred by using 1000 bootstrap iterations. Accessions numbers are shown in parentheses. Bootstrap values > 50% are shown at each node. Scale bars represent the nucleotide substitution percentage. Clone designations are shown by symbols: ●SuM , ■FIM and letters as G, M and K corresponding to the locations (Goa, Mangalore and Kochi) from where the clones were derived.
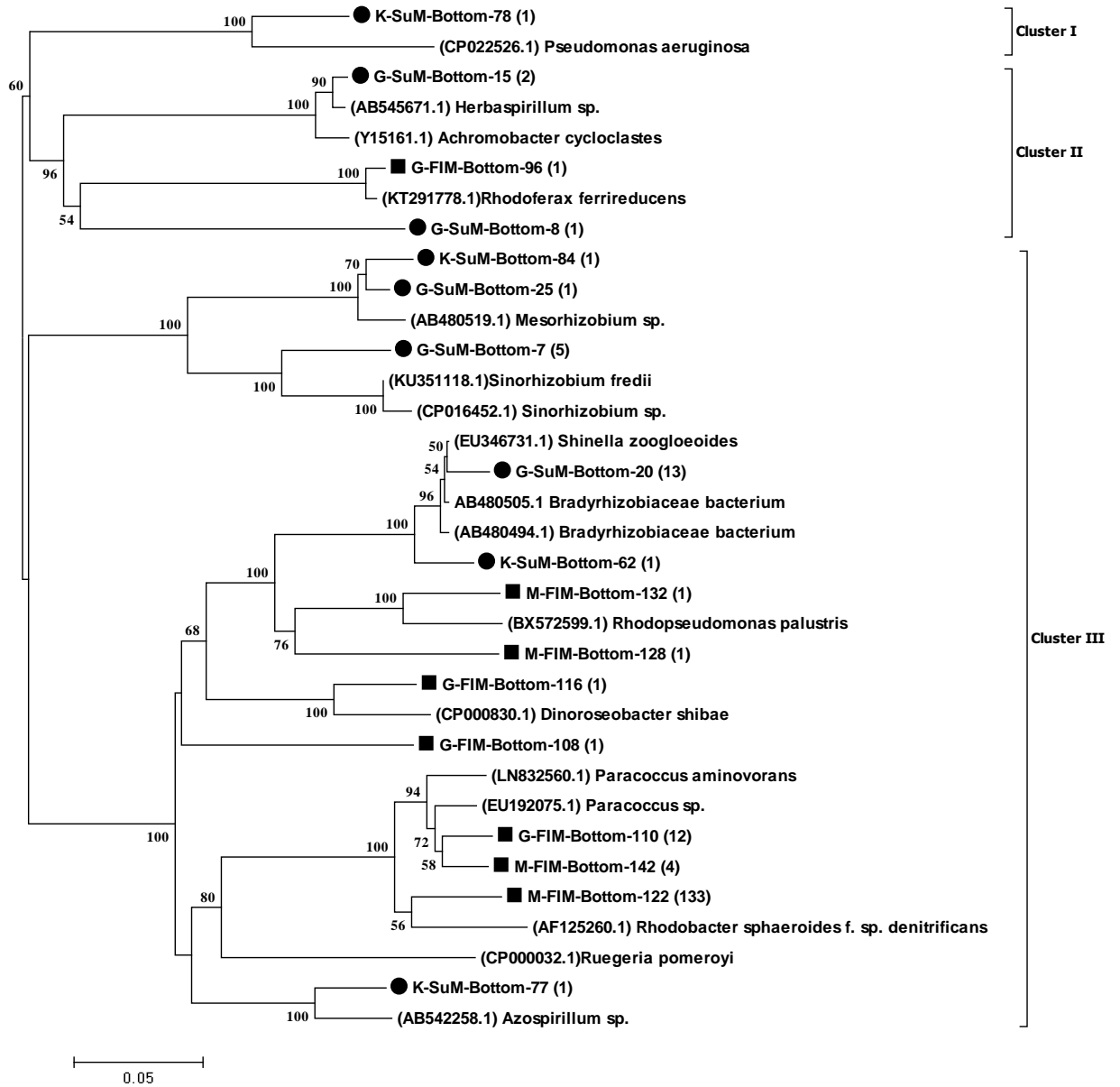
6.3.3 Statistical analyses of functional genes

The Chao1 estimator of species diversity, Shannon diversity index and Simpson diversity index and Goods coverage for each gene was calculated (Table 6.2). The order of richness of functional genes (number of estimated genotypes based on the Chao1 index) was *narG>nosZ>nirS* in SuM and FIM. The coverage of these clone libraries was 95 %, 98 % and 93 % for *narG*, *nosZ*, and *nirS* genes respectively. Rarefaction analysis also showed that *nirS* and *nosZ* reached saturation indicating better coverage during SuM and FIM. In general, *narG* was more in the number of bacterial types (Fig 6.5a and b).

Table 6.2: Spatiotemporal distribution of denitrifying functional genes, diversity indices and coverage of OTUs

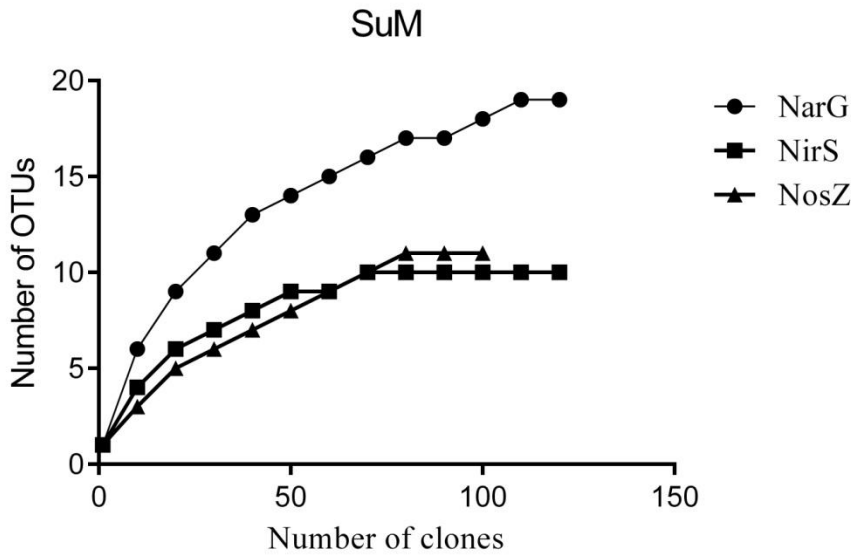| Gene	No. of clones	No. of OTUs	Shannon's index	Simpson's index	Chao1 index	Good coverage (%)
SuM						
NarG	120	19	2.23	0.17	24	95
NirS	120	10	1.51	0.34	10	98
NosZ	100	11	1.13	0.52	16	93
FIM						
NarG	180	24	1.44	0.51	25.90	96
NirS	150	5	0.43	0.82	5.00	98
NosZ	100	12	1.12	0.55	17.00	93

Fig.6.5a: Rarefaction curves of operational taxonomic units (OTU) of denitrifying genes (*nar,nir* and *nos*) obtained during SuM.
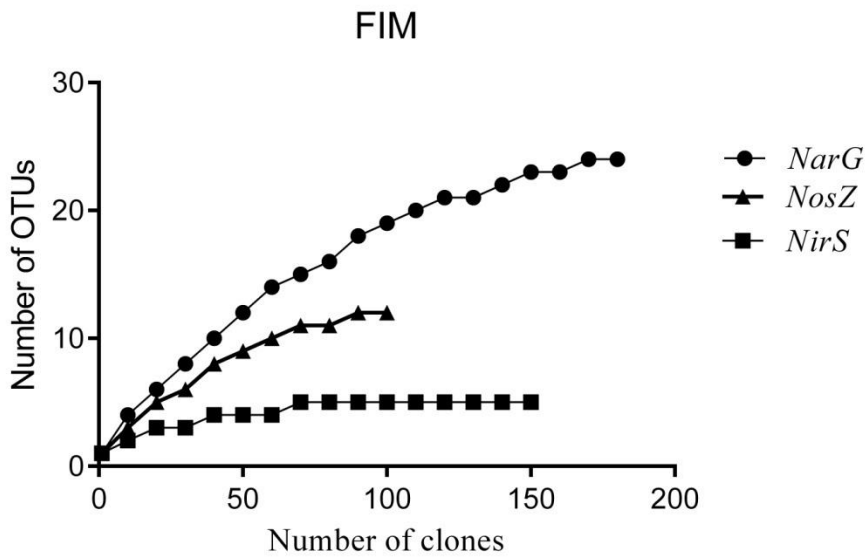

Fig.6.5b: Rarefaction curves of operational taxonomic units (OTU) of denitrifying genes (*nar,nir* and *nos*) obtained during FIM.

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6.4 Discussion

From the cooler (19-22°C) waters in the mid and near bottom depths, it is apparent that monsoon time (June-October) upwelled waters persisted during SuM and FIM. With the surface temperature in the range of 28-30°C during these periods, it can be suggested that the low saline freshwater cap as a result of intense rainfall (Pant and Rupa Kumar, 1977; DileepKumar, 2006) seems to prevent surfacing of upwelled water. During SuM the surface was well oxygenated, unlike the mid-depth and near-bottom waters. This is because hypoxia (with $O_2 < 1.4$ ml L^{-1} or 62.5 µM; 2 mg L^{-1}) (Levin et al. 2009) developed in the subsurface was due to upwelling (consumption of O_2 for degradation of organic matter as a result of higher biological productivity) and poor ventilation. The DO further reduced during FIM leading to suboxia because of stratification in addition to low oxic upwelled waters. With the collapse of upwelling, the water column is reported to become oxygenated during SIM (Jayakumar et al. 2004; Pratihary, 2007; Naqvi et al. 2010). Following the decline in DO concentrations, nitrate being the next abundant electron acceptor mediates the organic matter transformation. Observations in the Indian coastal waters since 1997 (Naqvi et al. 2000; Jayakumar et al. 2004) have shown that high nitrite concentrations (>1 µM) occur when the water is completely depleted of dissolved oxygen.

The DNA extracts from the low oxygen mid-depth and near-bottom waters containing high nitrate and nitrite concentrations could be amplified using the *narG* and *nirS* primers during SuM and FIM. But, *nosZ* amplification was positive only in the DNA extracts from samples collected from close to bottom during SuM and FIM. The failure to obtain *nirS* sequences from the oxygenated surface waters is perhaps an indication that denitrifying bacteria possessing *nirS* gene are rare in such layers (Jayakumar et al. 2013). There are no primer sets as yet for *nirS* gene displaying both complete coverage and specificity together.

Continuous addition of novel sequences would increase the repertoire on the diversity from the deoxygenated or oxygen losing regions. The fact that no novel target regions exist for *nirS* gene, it may not be possible to obtain a single ideal universal primer pair for each of the *nir* genes as reported by a recent study by Bonilla-Rosso et al. (2016).

Results of this study bring forth the fact that each denitrifying gene is specific to certain taxonomic groups. The 16s rRNA gene diversity of culturable as well as non-culturable bacteria (unpublished data) in this region is dominated by the phylum *Proteobacteria*. The same was evidenced in this study dominated by bacteria belonging to phylum *Proteobacteria*, indicating their significance in water column denitrification. This observation is consistent with previous studies of Heylen et al. (2006); Bru et al. (2007); Wyman et al. (2013) and Yu et al. (2014). Gene encoding for the membrane-bound nitrate reductase *narG* was distributed among taxonomically diverse bacteria, from *Alpha-*, *Beta-* and *Gamma-Proteobacteria*. The nitrate-reducing community is phylogenetically diverse and related to species of *Marinobacter, Halomonas, Shewanella, Pseudomonas, Pectobacterium, Stenotrophomonas, Alicycliphilus, Burkholderia, Delftia, Methylobacterium, Ochrobactrum, Brucella* as well as clones from soil were previously reported for nitrate reduction (Lee at al. 2009; Zhang et al. 2016). Also, the clones of *nirS* gene were closely related to *Pseudomonas stutzeri, Kocuria, Herbaspirillum* and *Alcaligenes* sp. This was consistent as also observed earlier in OMZs by Jayakumar et al. (2004) and Yu et al. (2014). Some of *nirS* were distantly related to the identified denitrifiers suggesting that denitrifiers possessing these gene sequences are yet to be isolated. In contrast to *narG* OTUs, the *nirS* OTUs decreased from SuM (hypoxic periods) to FIM (suboxic periods). Since it is likely that competition for carbon substrates and nitrate intensifies among denitrifiers themselves, the nutritionally versatile

groups become differentially abundant leading to decreased diversity at OTU levels. Thus, as also noted by Jayakumar et al. (2009, 2013), the observed dominance of a few types of denitrifying organisms, the denitrifier diversity decreased as denitrification progressed from initially low-oxygen-high-nitrate waters to low-oxygen-high-nitrite conditions. Bacteria harboring *nosZ* gene included *Pseudomonas, Herbaspirillum, Azospirillum, Bradyrhizobium, Sinorhizobium, Mesorhizobium, Shinella, Rhodopseudomonas, Rhodobacter, Paracoccus* sp. were also previously reported in paddy soil, wastewater and OMZ (Ishii et al. 2011; Hou et al. 2012; Yi et al. 2015).

In the present study, it was evidenced that the copy numbers of *narG* genes (10^7 L^{-1}) were higher than those of *nirS* and *nosZ* genes (10^6 L^{-1}) which were within the ranges reported by Wyman et al. (2013), Yu et al. (2014) and, Yi et al. (2015). Studies have reported the presence of *narG*, in order of 10^4 to 10^9 copies/ L, *nirS* genes as low as 10^3 copies/μl to 10^9 copies/L, and *nosZ* from 10^4 to 10^5 copies/L (Chon et al. 2011; Wyman et al. 2013; Jayakumar et al. 2013; Bourbonnais et al. 2014; Yu et al. 2014). The abundance of *nosZ* gene increasing northward from Kochi to Goa could be due to the receding upwelling intensity as has been reported by Naqvi et al. (2006; 2010). The qPCR data suggest seasonal differences in the abundance of denitrification communities in these coastal locations with the *narG* possessing assemblages being predominant during SuM and FIM.

Conclusion

Results of this study provide information on bacterial communities possessing genes for the denitrification. Quantitative data on the diversity, distribution, and abundance of nitrate, nitrite, and nitrous oxide reductase marker genes (*narG, nirS,* and *nosZ*) are key for evaluating their involvement in the removal of fixed nitrogen from the seasonal coastal

denitrifying region of the Arabian Sea. The distribution of these functional genes was consistent with the environmental conditions that define this coastal region. In essence, bacterial community structure varied seasonally in diversity and the composition of the assemblage reflecting the shift from hypoxia to suboxia. These results contribute to the understanding relationships between the abundance of genes, denitrifier community and their possible co-functioning in the nitrate, nitrite and nitrous oxide reduction (denitrification) process.

SUMMARY

Coastal upwelling systems are the most productive areas in the world's oceans. The west coast of India is located at the Eastern Arabian Sea upwelling system. Characterized by strong seasonal oscillations in physical forcing and biological production, the Arabian Sea is recognized as very productive of tropical oceanic regions. It is a region where the along-shore winds interact with the coastal topography to generate upwelling–downwelling dynamics (cross-shore Ekman transport) on the continental shelf. Strong south-west monsoon during summer months of June-October causes intense upwelling in its south eastern borders. Whereas, during the winter months (December-March), enhanced vertical mixing leads to surface cooling. Thus, the photic zone is replete with nutrients from subsurface layers during both these periods leading to generally high biological productivity anthropogenic and atmospheric input of nitrogenous and other nutrients. Higher consumption rates of dissolved organic matter lead to oxygen deficient conditions with dissolved oxygen concentrations reaching <1.42 ml L^{-1} (hypoxia or deoxygenation in the water column. In essence, depletion of oxygen necessitates bacteria to utilize nitrate via the denitrification process. In the denitrifying zone off the southwest coast of India, high concentrations of nitrite (> 15 µM) and nitrous oxide (> 500 nM) have been reported.

In the face of deoxygenation that induces enhanced denitrification, the hypoxic ecosystems would harbor unique, diverse communities of heterotrophic bacteria. Thus, such ecosystems are hotspots for oxygen-sensitive nitrogen transformations, where nitrate serves as the main terminal electron acceptor in the process of organic matter oxidation. In such cases, denitrification contributes to the removal of fixed nitrogen as N_2, with resulting impacts governing global nutrient cycles and in general, the climate system.

The following is an account of important observations/findings from this study.

- Sampling was carried out based on the oceanographic conditions as Spring Intermonsoon (SIM; March-May); summer monsoon (SuM; June-August) and Fall Intermonsoon (FIM; September-October) at the surface, mid-depth and bottom water Off Goa, Off Mangalore and Off Kochi. It was observed that during SuM and FIM seasons, the temperature and dissolved oxygen concentrations steeply decrease in the mid depth and bottom layers. Corresponding to this decrease, the nitrate and nitrite concentrations increase. However, constant temperature, and Dissolved Oxygen (DO) was constant throughout the water column during SIM and concentration of nitrogen species was null.

- Culture-dependent bacterial diversity by 16S rRNA gene sequencing from this study resulted in a total of 31 genera belonging to 5 phyla. *Alteromonas* and *Vibrio* spp. was predominant among the culturable bacteria. Distinct spatiotemporal variations in bacterial communities were observed. 24 different bacterial genera were observed Off Goa, 17 different bacterial genera were observed Off Mangalore and 13 different bacterial genera were observed Off Kochi. The diversity was in the order of Goa>Mangalore>Kochi. Low diversity was observed only during SuM.

- Culture-independent bacterial diversity was investigated based on 16S rDNA clone library approach and the clones were affiliated with *Proteobacteria* (includes *Alpha*, *Gamma*, *Beta* and *Delta* subdivisions), *Acidobacteria*, *Actinobacteria, Firmicutes*, *Bacteroidetes*, *Cyanobacteria*, *Marinimicrobia, Verrucomicrobia*., *Chloroflexi*,

Planctomycetes and *Omnitrophica bacterium*. Among the *Proteobacteria*, γ-*Proteobacteria* was predominant in this study area followed by *Cyanobacteria*.

- Both culture-dependent and culture-independent studies (i.e. 16S rRNA gene-based) have indicated that the phylum *Gammaproteobacteria* is the dominant phylum in all three sampled locations, independent of seasons.

- Phylogenetic groups *viz.*, *Proteobacteria (Beta* and *Delta* subdivisions), *Acidobacteria, Cyanobacteria, Marinimicrobia, Verrucomicrobia., Chloroflexi, Planctomycetes*and *Omnitrophicabacterium*obtained from non-culturable diversity were not detected in culture-dependent analysis.

- *Alteromonas, Idiomarina, Vibrio, Marinobacter, Pseudoalteromonas, Photobacterium, Halomonas, Pseudomonas, Erythrobacter* and *Bacillus* were the common genera observed in both culture-dependent and culture-independent analyses.

- Bacterial diversity was the least by both culture-dependent and culture-independent methods during Summer Monsoon

- Of the 900 cultures obtained from the study area, 673 cultures were nitrate reducers accounting for 75% of the total bacterial population in this study, which grouped into 42 different species.

- NRR ranged from 0.04–0.27 μMd^{-1} among all 42 cultures. The per cell NRR ranged from 0.41-2.40 $(X\ 10^{-14})$ mol cell^{-1}. It was seen that *Halomonas axialensis* recorded the highest NRR of 2.40 $(X\ 10^{-14})$ mol cell^{-1}. NRR was the least [0.41-0.57 $(X\ 10^{-14})$ mol cell^{-1}] by *Salinicola salarius* and *Microbacterium aquimaris*

- Selected 42 representative nitrate reducing cultures were checked for nitrous oxide (N_2O) production. N_2O production ranged from 7.00-119.32 nMd^{-1} among all 30

cultures. The per cell N_2O ranged from 0.29-5.54 (X 10^{-15}) mole cell^{-1}. Maximum N_2O production was by *Idiomarina sediminium* and *Marinobacter salsuginis* in the range of 5.18-5.54 (X 10^{-15}) mole cell^{-1}, followed by *Halomonas axialensis* [3.36 (X 10^{-15}) mole) cell^{-1}]. *Bacillus aryabhattai* produced the least N_2O [0.29 (X 10^{-15}) mole cell^{-1}.

- The nitrate reductase activities ranged among the tested cultures was low as 0.012-0.037 Uml^{-1} by *Micrococcus endophyticus, Vibrio tubiashii* and *Bacillus vietnamensis*. Notably, the highest activity of 0.084 and 0.119 U ml^{-1} was by *Idiomarina sediminium* and *Halomonas xianhensis* respectively.

- The 42 representative nitrate reducing cultures were checked for presence of denitrifying genes (*narG*, *nirS* and *nosZ*).

- The DNA extracts from the low oxygen mid-depth and near-bottom waters containing high nitrate and nitrite concentrations could be amplified using the *narG* and *nirS* primers during SuM and FIM. But, *nosZ* amplification was positive only in the DNA extracts from samples collected from close to bottom during SuM and FIM.

- Assemblages of bacteria possessing *narG, nirS* and *nosZ* indicated that complete denitrification could occur, particularly in the samples collected close to the bottom.

- Functional gene sequencing of bacterial communities brought out the fact that strains possessing these reductases were diverse and, dominated by members of *Alpha-, Beta-* and *Gamma-proteobacteria*, including taxonomic groups containing well known denitrifiers such as *Pseudogulbenkiania, Kocuria, Pseudomonas, Herbaspirillum, Achromobacter, Rhodoferax, Mesorhizobium, Sinorhizobium, Shinella, Bradyrhizobiaceae, Rhodopseudomonas, Dinoroseobacter, Paracoccus, Rhodobacter, Ruegeria* and *Azospirillum* sp.

- Besides the distinct spatiotemporal differences, the overall ranges of these three functional genes across the sampling sites ranged from 1.9×10^7 to 7.9×10^7 copies L^{-1} (*narG*), from 0.001×10^6 to 0.30×10^6 copies L^{-1} (*nirS*) and from 0.30×10^6 to 2.9×10^6 copies L^{-1} (*nosZ*).

FUTURE PROSPECTS

➢ To study the total bacterial diversity is a persisting challenge in any environment. To avoid the cloning bias, next generation sequencing (NGS) could reveal the total diversity in the coastal environment.

➢ Since various environmental factors regulate denitrification, analysis of mRNAs as an indicator of gene expression would determine the active population of bacteria performing denitrification.

➢ Improved isolation techniques to obtain bacterial cultures from oxygen depleted environment in anaerobic conditions.

➢ Microarray hybridization analyses of denitrifying genes.

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PUBLICATIONS

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• Poster presentation, International symposium on Microbial Response to Ocean Deoxygenation, (December 2-5, 2016), CSIR-NIO, Goa-India

•Poster presentation, New Perspectives in Biosciences, (December 7, 2017), Goa University, India